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Degree: *Doctor of Philosophy*

Year this Degree Granted: *2005*

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Identifying functional links between Nup53p,Nup170p and Kap121p. A role for kaps in NPC assembly and changes in the NPC during mitosis.

by

Charles Patrick Lusk

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

Department of Cell Biology

Edmonton, Alberta
Spring 2005

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Identifying functional links between Nup53p,Nup170p and Kap121p. A role for kaps in NPC assembly and changes in the NPC during mitosis,* submitted by **Charles Patrick Lusk** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

Dedication

To my parents,

for their unwavering support and encouragement.

Dedication

To my parents,

for their unwavering support and encouragement.

Abstract

Nuclear Pore Complexes (NPCs) form aqueous channels that fenestrate the nuclear envelope, and provide the sole sites of molecular exchange between the nucleus and the cytoplasm. The NPC is formed of proteins called nucleoporins, and we have investigated the functional interactions between the nucleoporin Nup53p and its binding partners. These include the nucleoporins Nup170p and Nic96p, in addition to a member of the β-karyopherin protein family, Kap121p. Nup53p interacts with Kap121p through a domain in its C-terminus that resembles an NLS, which we term the Kap121p-binding domain or KBD. We show that the interaction of Kap121p with the KBD is required for the efficient assembly of Nup53p into the NPC. This karyopherin-mediated function can also be performed by the Kap95p/Kap60p complex if the KBD is replaced by a classical NLS, suggesting a more general role for karyopherins in NPC assembly. Once within the context of the NPC, Nup53p exists in two states that are regulated in a cell cycle dependent manner. During interphase, Nup53p interacts with Nup170p through a region in its C-terminus that overlaps the KBD. Upon entry into G2/M-phase, molecular rearrangments occur within the NPC that break the interaction between Nup53p and Nup170p and expose the KBD. We provide evidence that the binding of Kap121p to Nup53p functions to inhibit Kap121p-mediated transport by slowing Kap121p import into the nucleus, and potentially driving premature cargo release. Constitutive activation of this inhibitory pathway by the overexpression of *NUP53* leads to a delay in mitotic progression. The M-phase specific molecular rearrangements occur concomitantly with the phosphorylation of Nup53p. We individually screened 119 out of 122 yeast kinases in an effort to identify the kinases responsible for these phosphorylation events. This

analysis revealed that Nup53p is a target of a casein kinase, Hrr25p. We demonstrate that Hrr25p directly binds Nup53p in an interaction that is destabilized by phosphorylation. The functional significance of this may be reflected *in vivo* as Hrr25p does not associate with Nup53p during mitosis. We also provide evidence that Nup53p is a target of the mitotic kinase Cdk1p. The mutation of Cdk1p phosphorylation sites witin Nup53p impacts on Nup53p function, as the resulting *nup53* allele cannot fully complement *NUP53* synthetic lethal interactions.

Acknowledgements

It has been my priviledge to be surrounded by a number of talented individuals who have guided and supported me through this work. Foremost of these is Rick Wozniak. Thank you Rick for sharing your passion for science, and for taking the time to create an environment where my own passion could be nurtured. I am grateful that you pushed me to set challenging goals, but more importantly, that you endowed me with the skills and knowledge so that I could attain them. It has been a great honor to work so closely with you over the years and I will greatly miss you and your lab. I am very excited, however, about the future you helped establish for me and look forward to sharing my continued successes, and yours, in the years to come.

I have been fortunate to work in an environment where there is little distinction between colleagues and friends. Taras Makhnevych made integral contributions to this work for which I am most grateful. It was a great pleasure developing and testing our nuclear transport models, and I hope that we will have the opportunity to work together again. Marcello Marelli, Lisa Hawryluk-Gara, Robert Scott and Andrea Anderson helped by filling my picture albums with some of my fondest memories. You have been a tremendous reservoir of support and friendship over the years, and my time here would have been dreadfully dull without all of you. Lisa, thanks for showing me that there's another way and Rob, you are not that easy to forget!

I expect great things from all of you.

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List of Abbreviations

5-FOA.....5'-fluoroorotic acid
αF.....α-factor
APC.....anaphase promoting complex
ATP.....adenosine triphosphate
Ben.....benomyl
bp.....base pair
BP.....broad pass
°C.....degrees celsius
CK.....casein kinase
cNLS.....'classical' NLS
Cdk.....cyclin dependent kinase
Da.....Dalton
DNA.....deoxyribonucleic acid
ECL.....enhanced chemoluminescence
EM.....electron microscopy
Fig. .....Figure
FG.....phenylalanine, glycine
g.....gram
g.....gravitational force
Gal.....galactose
G1.....gap one phase
G2.....gap two phase
GAP.....GTPase activating protein
GDP.....guanosine diphosphate
GEF.....guanosyl exchange factor
GFP.....green fluorescent protein
GFP^+.....enhanced green fluorescent protein
Glc.....glucose
GST.....glutathione-S-transferase
GT.....glutathione
GTP.....guanosine triphosphate
GTPase.....guanosine triphophatase
h.....hour
HA..... ....Hemagluttinin
hnRNP.....heterogenous nuclear RNP
HU.....hydroxyurea
HRP.....horseradish peroxidase
IgG.....immunoglobulin G
INM.....inner nuclear membrane
IPTG.....isopropyl-β-D-thiogalactoside
kap.....karyopherin
LP.....long pass
min.....minutes
μ.....micro

m.....milli
M.....Mitotic phase/moles per litre/Mega ($1x10^6$)
n.....nano
mRNA.....messenger RNA
NE.....nuclear envelope
NEBD.. .....nuclear envelope breakdown
NES.....nuclear export signal
NLS.....nuclear localization signal
Noc.....nocodazole
NPC.....nuclear pore complex
nup.....nucleoporin
OD.....optical density
ONM.....outer nuclear membrane
ORF.....open reading frame
pA.....protein A
PAGE.....polyacrylamide gel electrophoresis
PCR.....polymerase chain reaction
pH.....$-\log[H^+]$
PM.....pore membrane
pom.....pore membrane protein
RFP.....red fluorescent protein
RNA.....ribonucleic acid
RNAi.....RNA interference
RNP.....ribonucleoporin
S.....Synthesis Phase
SDS.....sodium dodecyl sulphate
SM.....synthetic media
snRNP.....small nuclear RNP
SPB.....spindle pole body
TCA.....trichloracetic acid
tRNA.....transfer RNA
ts.....temperature sensitive
YPD.....yeast extract peptone and dextrose
YP-gal.....yeast extract peptone and galactose
YFP.....yellow fluorescent protein

Chapter I: *Introduction*[1]

[1] Portions of this chapter are similar to a published work: Lusk et al., 2004, Biochemistry and Cell Biology, 82, pp 618-25. Permission for their reproduction is granted by the NRC Research Press copyright policy.

1.1 Preface

In eukaryotic cells, the contents of the nucleus are physically separated from the cytoplasm by an impermeable double membrane called the nuclear envelope (NE). This separation of the contents of the nucleus, including the cell's chromatin and transcriptional machinery, from the cytoplasm, containing the cell's translational machinery, demands that cells regulate a vast array of macromolecular traffic between these compartments. All this traffic occurs through nuclear pore complexes (NPCs), which extend across the NE and act as gatekeepers for all transport. The NPCs function in concert with a family of soluble factors, termed karyopherins or kaps (also termed importins or exportins) that recognize cargos by binding a nuclear localization signal (NLS) or a nuclear export signal (NES) in either the cytoplasm or nucleus, respectively, and docks them to the NPC for subsequent translocation.

Individual karyopherins have the ability to bind specific groups of cargos, thus providing the cell with a means to independently regulate the transport of different classes of molecules including most proteins and ribonucleoprotein particles between the cytoplasm and the nucleoplasm. Understanding the regulatory mechanisms that underlie these pathways is paramount to understanding normal cellular function, including how cells regulate gene expression, progress through the cell cycle, transduce both intra- and extra-cellular signals, and how viruses gain access to the nucleus for productive infection.

1.2 The Nuclear Envelope

As the defining feature of eukaryotic cells, the nucleus is delimited by a double membrane lipid bilayer called the nuclear envelope (NE) that compartmentalizes the

DNA and transcriptional machinery, physically isolating it from the rest of the cell. The NE is composed of three functionally distinct membrane systems that are defined by specific biochemical complements of proteins: the inner and outer nuclear membrane (INM/ONM) and the pore membrane (PM)(Holaska et al., 2002; Mattaj, 2004). The ONM is indistinguishable from the endoplasmic reticulum and is replete with actively translating ribosomes. In contrast, the INM maintains a unique complement of proteins which interface with an intranuclear meshwork of laminar protein fibres called the nuclear lamina (Hutchison, 2002). The lamina forms an inner shell under the INM that supports nuclear structure and it, along with other INM proteins, make contact with the underlying chromatin (Taddei et al., 2004). At points along the INM and ONM these membranes fuse to form the PM. The PM creates transcisternal channels across the NE that are occupied by large protein assemblies called NPCs.

1.3 Nuclear Pore Complexes

NPCs provide an aqueous channel that allows the passive diffusion of ions and metabolites, but is selective in its transport of macromolecules. To accommodate a bulk transport which has been measured at 80 MDa/NPC/second, the NPC structure is correspondingly massive, and has been estimated to be as large as 125 MDa in vertebrates, and 72 MDa in yeast (Reichelt et al., 1990; Rout and Blobel, 1993; Ribbeck and Gorlich, 2001). These masses likely represent upper limits, as more conservative estimates of ~60 MDa and ~44 MDa for vertebrate and yeast NPCs have been proposed by analyzing the relative abundance of all nucleoporins (Rout et al., 2000; Cronshaw et al., 2002). Nonetheless, the relative size difference between yeast and vertebrate NPCs is

reflected in their physical dimensions, particularly with respect to the length of the transport channel which corresponds to the greater lumenal thickness of the vertebrate nuclear envelope (Yang et al., 1998; Fig. 1-2). While these structures clearly diverged, their overall structure and function has remained remarkably conserved throughout evolution (Yang et al., 1998; Stoffler et al., 1999; Vasu and Forbes, 2001; Cronshaw et al., 2002; Kiseleva et al., 2004; Suntharalingam and Wente, 2003 and see 1.3.1.5).

Our understanding of NPC structure has evolved from numerous EM studies of both yeast and vertebrate NPCs (Reichelt et al., 1990; Jarnik and Aebi, 1991; Hinshaw et al., 1992; Akey and Radermacher, 1993; Yang et al., 1998; Stoffler et al., 2003; Kiseleva et al., 2004; Beck et al., 2004). As early as 1950, "annuli" or "rings" were observed decorating the surface of *Xenopus* oocyte nuclear envelopes, and nine years later the term "nuclear pore complex" was proposed as a name for these protein assemblies (Callan and Tomlin, 1950; Watson et al., 1959). Since then, EM-studies have revealed an elaborate structure that consists of a central core assembly that is embedded in the NE, off of which emanate both cytoplasmic and nuclear specific filaments (Fig. 1-1). The "cytoplasmic filaments" reach out into the cytoplasm and provide the initial docking sites for importing molecules. They are generally thought to be flexible, and recent data suggests that they have a "kink" that points the filaments towards the central transporter (Beck et al., 2004). On the nuclear side, extending from the central core are nuclear filaments that form into a more structured unit that resembles a basket. While also serving as a docking site for molecules in transit, functional links have been described between components of the nuclear basket and the underlying nuclear lamina, as well as with chromatin (Smythe et al., 2000; Galy et al., 2000; Feuerbach et al., 2002; Ishii et al., 2002).

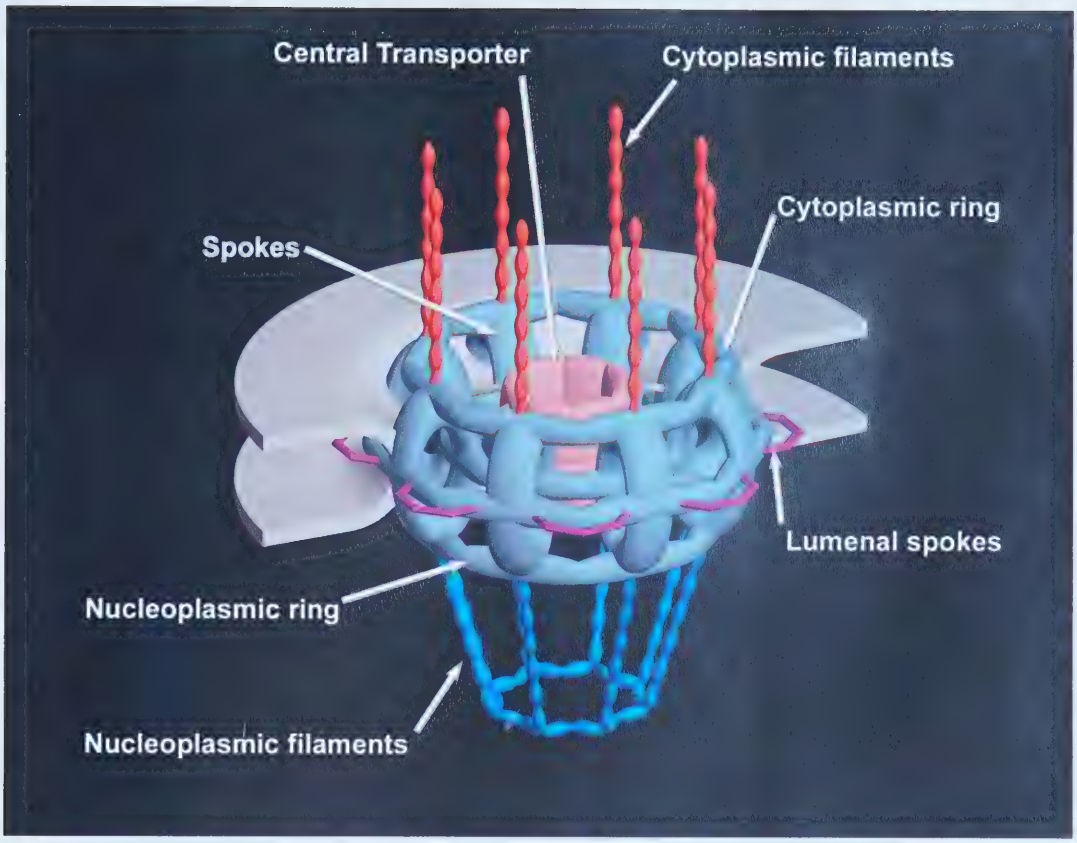

Figure 1-1. A model of the vertebrate NPC.

A schematic of the NPC within a cross section of the nuclear envelope (grey). The NPC is constructed with a central core consisting of ring structures (light blue and purple) from which eight spokes radiate to surround a central transporter (pink). Extending from the core into the cytoplasm and nucleoplasm, are cytoplasmic filaments (red) and a nuclear basket (blue). Adapted from Rout and Aitchison, 2001.

Unlike the peripheral filaments, the central core of the NPC is markedly symmetrical in planes both parallel (2-fold) and perpendicular (8-fold) to the nuclear envelope. It spans the width of the NE and is composed of an elaborate spoke-ring complex that surrounds the central transport channel (Fig. 1-1). Imaging the transport channel has proven to be more controversial. Molecules in transit occlude the opening and often the peripheral filaments collapse onto the channel during fixation (Jarnik and Aebi, 1991; Akey and Radermacher, 1993; Stoffler et al., 2003; Beck et al., 2004). Nonetheless, numerous studies, including those using Atomic Force Microscopy, have consistently hinted that there are physical changes that occur to the channel during transport (Akey and Radermacher, 1993; Akey, 1995; Kiseleva et al., 1998; Rakowska et al., 1998; Stoffler et al., 1999; Goldberg et al., 2000; Jaggi et al., 2003) . These observations have prompted the suggestion that the NPC is gated or that it can take on different conformational states. Perhaps the best evidence for this phenomenon comes from a recent study which took advantage of a relatively benign form of fixation that allowed the visualization of transport competent NPCs at unprecedented resolution (Beck et al., 2004). This study provided compelling evidence that the NPC can exist in two structural states, that strongly hint that the NPC is not just a benign transport channel, but may have dynamic properties that potentially function to move molecules through the NPC (Beck et al., 2004). The mechanisms that drive these changes and whether they play a direct role in nuclear transport, still remain to be understood.

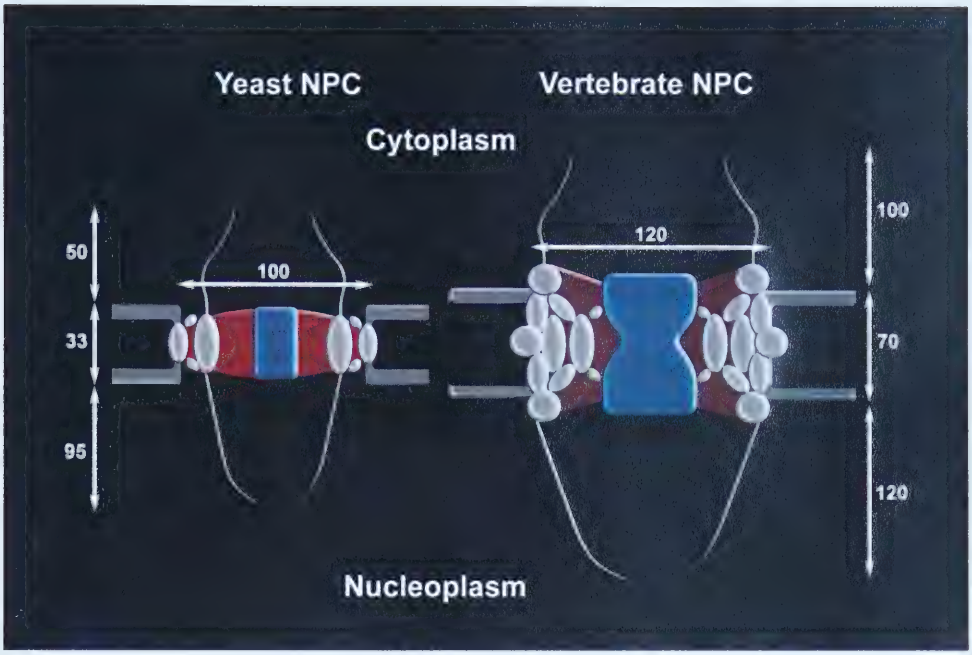

Figure 1-2. Comparison of yeast and vertebrate NPCs.

General schematics of the yeast and vertebrate NPC shown in cross section, embedded in the nuclear envelope (dark grey). The central framework and peripheral filaments are represented in silver and the central transporter in blue. The vertebrate NPC is, in general, ~15% larger than its yeast counterpart. Numbers are in nm. Modified from Yang et al., 1998.

1.3.1 Nucleoporins

The NPC is constructed of proteins called nucleoporins, or nups. Early efforts to define the nup complement have relied on traditional biochemical and genetic approachs (Doye and Hurt, 1997), and they have culminated with two comprehensive proteomic analyses of purified fractions of both the rat and yeast NPCs (Rout et al., 2000; Cronshaw et al., 2002). Remarkably, despite their apparent mass, these studies revealed that both the yeast and vertebrate NPC are constructed with only ~30 proteins. In a broad context, these nups can be grouped into four categories. The first category consists of the pore-membrane associated proteins, or poms. They represent those nups that insert into the pore membrane and likely anchor the NPC in the NE. The second and third categories are identified by the presence or absence of a number of peptide signatures that contain, at their core, a repetitive motif of phenylalanine and glycine amino acid residues (FG). Appropriately they are named the FG or non-FG nups. Lastly, there is a a group of FG-nups that are not anchored constituents of the NPC, and are able to shuttle between the nucleus and the cytoplasm. These will be referred to as the mobile nups.

1.3.1.1 Poms

There are three poms in yeast, Pom34p, Pom152p and Ndc1p and two, pom121 and gp210, in vertebrates. None of these proteins are homologues. Regardless, they are all thought to anchor NPCs to the pore membrane and have roles in NPC assembly (Gerace et al., 1982; Wozniak et al., 1989). In vertebrates, a number of studies have investigated the temporal integration of pom121 and gp210 into NPCs during NPC reassembly after mitosis, or in *Xenopus in vitro* cell free assays (Bodoor et al., 1999;

Daigle et al., 2001; Antonin et al., 2005). These studies have revealed that pom121 is incorporated at an early stage in NE biogenesis when core NPC constituents are not in place, whereas gp210 appears to be recruited much later. As these data suggest, pom121 likely plays a critical role in driving early events in NPC assembly. To support this idea, depletion of pom121 from *Xenopus* nuclear reconstitution assays prevents the fusion of chromatin attached vesicles that form the nascent NE. Moreover, this block in NE formation can be alleviated by the co-depletion of the Nup107-160 complex, providing a functional connection between NE formation and NPC assembly (Antonin et al., 2005). While gp210 is completely dispensable for these initial events in NPC assembly, its depletion in *C. elegans* embryos suggests that it may partially function in determining the proper distribution of NPCs on a fully formed NE (Cohen et al., 2003).

Consistent with the idea that poms function in early NPC assembly events, there is evidence supporting a similar function for the yeast poms. The overproduction of the FG-nup Nup53p results in a dramatic intranuclear proliferation of the NE that order into lamellar stacks (Marelli et al., 2001). The lamellar membranes have transcisternal pores similar to those found on the NE except that they are devoid of NPCs. In addition to Nup53p, only two poms, Ndc1p and Pom152p, appear on these membranes, strongly suggesting that they may be involved in the formation of these pre-pores (Marelli et al., 2001). Furthermore, certain mutants of *NDC1* have been shown to have defects in the incorporation of specific nups into the NPC (Lau et al., 2004) Ndc1p is unique in that it is also a shared component of both the NPC and the spindle pole body, the yeast microtubule organizing centre (Chial et al., 1998).

1.3.1.2 FG-nups

Of the 30 nups, approximately one third contain FGs and they play a direct role in nuclear transport. Consistent with this idea, mutations in certain FG-nups negatively influence the transport of a number of different substrates (Fabre and Hurt, 1997). These effects are manifested because of the direct interactions between the FG-nups and the soluble transport machinery, specifically the karyopherins (Radu et al., 1995; Kraemer et al., 1995; Iovine et al., 1995; Rexach and Blobel, 1995; Aitchison et al., 1996; Hu et al., 1996; Morianu and Blobel, 1997; Iovine and Wente, 1997; Delphin et al., 1997; Rout et al., 1997; Yaseen and Blobel, 1997; Marelli et al., 1998; Shah et al., 1998; Booth et al., 1999; Seedorf et al., 1999; Solsbacher et al., 2000; Denning et al., 2002; Allen et al., 2001; Hood et al., 2000; Ben-Efraim and Gerace, 2001; Gilchrist et al., 2002; Pyhtila and Rexach, 2003). Numerous studies have documented these interactions, and it is the FG-repeats themselves that provide the binding sites for the karyopherins (for a review, see Ryan and Wente, 2000). The best highlight of the importance of this interaction in nuclear transport is provided by the solved tertiary structures of Kap-β1 in complex with an FxFG and a GLFG-peptide (Bayliss et al., 2000; 2002). These studies reveal direct contacts between the phenylalanine residues of the FG peptides and the Kap-β1 backbone. Perhaps more importantly, mutating a critical contact residue on Kap-β1 not only reduces its affinity for FG-nups but inhibits the nuclear import of an NLS reporter, providing direct evidence of the importance of FGs as major facilitators of nuclear transport (Bayliss et al., 2000).

It has been estimated that there are ~128 FG-domains per NPC, many of which are thought to occur in up to 16 copies (Rout et al., 2000, Strawn et al., 2004). Thus, the

NPC is literally lined with thousands of potential kap binding sites. Furthermore in yeast, of all three groups of nups, only certain FG-nups are asymmetrically localized on either the cytoplasmic or nuclear side of the NPC (Rout et al., 2000). Both peripheral elements, the cytoplasmic filaments and the nuclear basket are likely composed solely of FG-containing nups (Stoffler et al., 1999; Rout et al., 2000; Vasu and Forbes, 2001; Suntharalingam and Wente, 2003). As discussed in section 1.3, these structures, particularly the cytoplasmic filaments, are considered to be flexible. In support of this idea, an analysis of the primary structure of nups reveals that FG-nups are enriched in disordering amino acid residues, and can adopt 'natively unfolded' conformations (Denning et al., 2002; 2003). The potential significance of disorder in the pore is likely intimately linked with the mechanistic aspects of the NPCs role in nuclear transport, and will be discussed in greater detail in section 1.5.

1.3.1.3 Non-FG-nups

Functions for the non-FG nups are not as well defined, although they likely have integral roles in NPC assembly and organization. They are also proposed to provide the scaffold on which the FG-nups are organized (Suntharalingam and Wente, 2003). Consistent with this idea, the Nup107-160 complex which consists largely of non-repeat nups, has been shown to play a central role in NPC formation. The incorporation of a number of FG-nups, for example, is inhibited by the depletion of two nups, Nup107 and Nup133, of this complex by RNAi (Boehmer et al., 2003; Walther et al., 2003). Moreover, the role that this complex plays in NPC assembly can be dramatically visualized in *Xenopus in vitro* nuclear reconstitution assays where the depletion of the

Nup107-160 complex completely abolishes NPC formation on a nascent NE (Walther et al., 2003; Harel et al., 2003). The yeast counterpart of this complex, the Nup84p complex, likely functions in a similar manner as mutations in complex members affect NPC formation and their distribution along the NE (Aitchison et al., 1995b; Siniossoglou et al., 1996; Siniossoglou et al., 2000; Bai et al., 2004). Furthermore, *in vitro* pulldown experiments using immobilized recombinant GST-fusions of FG-nups indicate that they are able to interact with the Nup84p complex, underscoring the Nup84p-complex function as a structural unit that may help support the transport interface (Allen et al., 2002, and see section 1.3.1.5).

Other examples of non-FG nups include Nic96p and its vertebrate orthologue Nup93, both of which are required for proper NPC assembly, and Nup170p, which also appears to be a central player in organizing the FG-nups (Zabel et al., 1996; Grandi et al., 1997; Galy et al., 2003). In the absence of Nup170p, there are changes in the localization and stoichiometry of several FG-nups including Nup1p, and Nup2p (Kenna et al., 1996). Moreover, there appears to be a significant destabilization of NPCs lacking Nup170p, as many FG and non-FG nups alike are dissociated from *nup170Δ* NPCs in the presence of aliphatic alcohols or energy poisons (Shulga and Goldfarb, 2003). These effects also seem to manifest themselves by increasing the size of the diffusion channel through the NPC, an effect mimicked in *nup188Δ* strains (Shulga et al., 2000). Importantly, there are physical and genetic interactions between the non-FG and FG-nups, reinforcing the idea that while the non-repeat nups may not directly interact with kaps, they are nonetheless vital for nuclear transport (Doye and Hurt, 1997).

1.3.1.4 Mobile nups

While the vast majority of nups are considered to be stable constituents of the NPC, an analysis of the relative turnover of nups in vertebrates indicates that Nup50 and Nup153, and to a lesser extent, gp210, dynamically associate with the NPC (Daigle et al., 2001; Rabut et al., 2004; Griffis et al., 2004). Similar studies have revealed that Nup98 is also able to move on and off the NPC (Griffis et al., 2002; 2004). While these experiments were performed with overproduced GFP-tagged nups, these data support other studies where endogenous Nup50 and Nup153, and in yeast, Nup2p, have been shown to shuttle between the nucleus and cytoplasm (Nakielny et al., 1999, Dilworth et al., 2001; Lindsay et al., 2002). The mobility of these proteins is linked to intimate interactions between these nups and the soluble transport machinery. Nup153, for example, shuttles because of its interaction with Kap-β2 through a region in its N-terminus that resembles an M9-NLS (Nakielny et al., 1999). The association of Nup2p and Nup50 with the NPC appears to be regulated by both kaps and Ran-GTP (Dilworth et al., 2001; Denning et al., 2001; Lindsay et al., 2002). Functional roles for nup mobility, however, have yet to be clearly established. There is evidence to suggest that Nup50 acts as a cofactor in Kap-α/Kap-β1–mediated import by stabilizing the interaction between the import complex (Lindsay et al., 2002). A similar function has been suggested for Nup153 in nuclear export (Nakielny et al., 1999). In addition, the mobility of Nup2p has been proposed to help clear kap-cargo complexes from the nuclear basket to help support efficient nuclear import (Dilworth et al., 2001). In a broad context, the existence of these nups supports the idea that the NPC provides a dynamic interface for nuclear transport reactions, in both yeasts and vertebrates.

1.3.1.5 Conservation and organization

As described above, all four of these nup classes form both the vertebrate and yeast NPC. Furthermore, both the yeast and vertebrate NPCs are composed of the same number of proteins that construct similar structures (see section 1.3). Despite these observations, the degree to which individual nups are conserved is variable (Stoffler et al., 1999; Vasu and Forbes 2001; Cronshaw et al., 2002; Suntharalingam and Wente, 2003). There are documented examples where vertebrate nups can functionally replace their yeast counterpart, but this has not been extensively tested for many conserved nup orthologues (Aitchison et al., 1995b; Strahm et al., 1999). There is, however, a remarkable conservation of nup interactions. In both yeast and vertebrates, certain nups exist in defined subcomplexes that are proposed to make up the modular units that form the NPC (Vasu and Forbes, 2001; Cronshaw et al., 2002; Suntharalingam and Wente, 2003). As a case in point, the 7 member 'Nup84p complex' has been purified from yeast and also reconstituted *in vitro* from recombinant proteins (Siniossoglou et al., 1996; Siniossoglou et al., 2000; Lutzmann et al., 2002). By EM this complex was imaged in a "Y" shape and it has been suggested that it forms part of the central scaffold of the NPC (Allen et al., 2002; Belgareh et al., 2001; Harel et al., 2003). The similarity of these complexes also suggests that they have conserved functional roles. Using the yeast Nup84p complex again as an example, it and its vertebrate counterpart, the Nup107-160 complex, both appear to be essential for proper NPC assembly (Siniossoglou et al., 1996; 2000; Boehmer et al., 2003; Walther et al., 2003; Harel et al., 2003).

Other subcomplexes include the Nsp1p complex in yeast, and its vertebrate counterpart, the Nup62 complex (Dabauvalle et al., 1990; Finlay et al., 1991; Kita et al., 1993; Grandi et al., 1993; Grandi et al., 1995; Guan et al., 1995; Hu et al., 1996; Schlaich et al., 1997; Bailer et al., 2001). This complex appears to have integral roles in nuclear transport, including RNA export (Grandi et al., 1995; Levesque et al., 2001; Bailer et al., 2001). Lastly, there is the vertebrate Nup93 complex, which contains Nup93, Nup188, Nup205 and Nup53 (Grandi et al., 1997; Miller et al., 2000; Hawryluk-Gara et al., 2005). The yeast equivalent of this complex has yet to be uncovered, however, Nup53p interacts with Nic96p (vertebrate Nup93) in immunopurifications and by two-hybrid analysis (Fahrenkrog et al., 2000; S.E. Tcheperegine and R.W. Wozniak, unpublished; Chapter IV and Appendix). In addition, other two hybrid data have revealed an interaction between Nup53p and Nup192p (vertebrate Nup205)(Uetz et al., 2000). Taken together, these data likely point to the existence of this complex in yeast as well. Interestingly, Nup53p is also found in complex with Nup170p and Nup59p, which serves to illustrate that certain nups cannot be easily classified into individual interacting modules (Marelli et al., 1998; Hawryluk-Gara et al., 2005). These observations suggest that certain nup-nup interactions may be regulated, or that the interaction of a nup with different subcomplexes may contribute to specific functional roles.

1.4 The soluble transport machinery

The NPC controls constitutive transport by two basic mechanisms: (1) it acts as a passive diffusion barrier to molecules <9 nm in diameter and (2), through a more widely studied pathway, it mediates macromolecular traffic using kaps and other soluble factors

including the small GTPase Ran. A broad spectrum of macromolecules cross the NE, including mRNAs, tRNAs, ribosomal proteins, ribosomal subunits, snRNPs and many soluble proteins. These various classes of molecules contain different nuclear localization or nuclear export signals. In most cases, they are recognized by different members of a family of structurally related proteins collectively referred to as karyopherins, but there are also other soluble transport factors with dedicated roles in specific transport pathways, including the export of mRNA.

1.4.1 Karyopherins

There are 15 kaps in yeast and upwards of 22 in vertebrates that function primarily as either importers and exporters, although at least one appears to function in both capacities (Wozniak et al., 1998; Gorlich and Kutay, 1999; Strom and Weis, 2001; Macara, 2001; Fried and Kutay, 2003; Yoshida and Blobel, 2001). The karyopherin family is segregated into two functionally related groups based on their similarity to the first two kaps identified, Kap-β1 and Kap-α. Appropriately, they are collectively referred to as either β-kaps or α-kaps. There is only one α-kap in yeast and six in vertebrates, and they act as adaptor molecules for Kap-β1, by bridging the interaction between Kap-β1 and NLS-containing proteins (Goldfarb et al., 2004). The vast majority of the β-kaps, however, are thought to directly recognize their cargos.

With the exception of a region in their N-termini that mediates an interaction with Ran-GTP, the sequence similarity between members of the β-kap family is relatively low (Wozniak et al., 1998; Gorlich and Kutay, 1999; Fried and Kutay, 2003). Nonetheless, β-kaps are presumed to adopt a similar tertiary structure (Fukuhara et al., 2004; and see

references below). Consistent with this idea, crystal structures of both Kap-β1, Kap-β2, Crm1 and Cse1p have been solved revealing similar structures constructed by a series of HEAT repeats (Cingolani et al., 1999; Chook and Blobel, 1999; Vetter et al., 1999; Matsuura and Stewart, 2004; Petosa et al., 2004). As a structural unit the HEAT repeat is a stretch of ~40 amino acid residues that forms two α-helices connected by a short linker (Andrade and Bork, 1995). The α-helices stack onto one another and endow the polypeptide with a superhelical twist, which in turn, suggests significant flexibility in the molecule. This fluidity is likely required to allow the kaps to interact with multiple components of the transport machinery, including signal sequences, nups and Ran-GTP. In support of this notion, Kap-β1 adopts a number of different conformations that function mechanistically to either break or stabilize its interactions with these proteins (Bayliss et al., 2000; Vetter et al., 1999; Chook and Blobel, 1999; Cingolani et al., 2000; Nevo et al., 2003; Chook et al., 2002). Furthermore, the elucidation of these structures has revealed that there are two FG-peptide binding sites in N- and C-terminal regions of the proteins. This might suggest that kaps can travel through the NPC by making simultaneous contacts with multiple FG nups (Kuersten et al., 2002; Bednenko et al., 2003).

1.4.2 Signal sequences

There are thousands of molecules that must be carried through the NPC by karyopherins. All of these molecules harbor signal sequences that are directly recognized by kaps. Based on their final cellular destination, these signals are categorized as nuclear localization or nuclear export signals (NLSs and NESs). The majority of these signals do

not fall into a canonical consensus and predicting them has proven to be extremely difficult (Fried and Kutay, 2003). For some, this is likely because these signal sequences retain a degeneracy so that they can be recognized by more than one karyopherin. For example, Kap121p and Kap104p both appear to recognize an NLS rich in arginine and glycine amino acid residues (RG-NLS), and Kap104p can functionally substitute for Kap121p in the import of an RG-NLS-containing nucleolar protein, Nop1p (Leslie et al., 2004). Another example is the import of histones which requires the concerted effort of at least three kaps, Kap114p, Kap121p and Kap123p (Mosammaparast et al., 2001). These examples reflect a functional redundancy that exists between the kaps, and may help explain the observation in yeast, that deletions of some kap genes are lethal while others are not (Wozniak et al., 1998; Jans et al., 2000). There are, however, NLSs and NESs that conform to a consensus sequence that is recognized by only one kap. A good example is the "classical" NLS which is recognized by the Kap-β1/Kap-α heterodimer (Fried and Kutay, 2003). The specificity between a kap and NLS may serve to define distinct transport pathways, and invoke the possibility that these pathways can be independently regulated to direct the transport of specific subsets of molecules.

1.4.3 Ran

In addition to the kaps, nucleo-cytoplasmic exchange requires the activity of the GTPase Ran (Moore, 1998; Gorlich and Kutay, 1999; Macara, 2001; Weis, 2003; Fried and Kutay, 2003). Ran provides the only known energy source required for maintaining the transport cycle, which is elicited from a steep potential energy gradient across the NPC. This gradient is formed by the activity of a number of proteins. First, Ran is

imported into the nucleus by a dedicated transport factor, Ntf2p, which maintains the nuclear concentration of Ran at a 200 to 500 fold ratio over its cytoplasmic counterparts (Smith et al., 2002; Kalab et al., 2002; Gorlich et al., 2003). Second, upon nuclear entry, Ran encounters a chromatin-bound Ran-GEF activity which converts the majority of nuclear Ran into a GTP-bound state (Ohtsubo et al., 1989; Bischoff and Ponstingl, 1991; Nemergut et al., 2001). Third, the cytoplasmic pool of Ran is maintained in a GDP-bound form by a Ran GTPase activating protein (Ran-GAP) which is primarily cytoplasmic, much of which is bound to the cytoplasmic face of the NPC through Nup358/RanBP2 (Matunis et al., 1996; Mahajan et al., 1997; Saitoh et al., 1997). The resulting gradient of Ran-GTP formed by these proteins contributes to the directionality of transport. To emphasize this point, the Ran gradient can be artificially reversed to allow the import of NES-containing proteins (Nachury and Weis, 1999).

The Ran-GTP gradient functions by triggering the assembly and disassembly of transport complexes in the correct compartments (Fig. 1-3; Gorlich and Kutay, 1999; Macara, 2001; Weis, 2003; Fried and Kutay, 2003). For example, the formation of an import complex between a kap and cargo is stable in the presence of cytoplasmic Ran-GDP. However, once the kap-cargo complex traverses the NPC and enters the nucleoplasm, Ran-GTP binds to the kap, displacing its cargo and terminating import (Fig. 1-3; Gorlich and Kutay, 1999; Macara, 2001; Weis, 2003; Fried and Kutay, 2003). Interestingly, NTP hydrolysis is not required for translocation through the NPC or for the dissociation of cargo in the nucleus, indicating that an individual import event does not require energy (Schwoebel et al., 1998; Englmeier et al, 1999; Ribbeck et al., 1999) Energy is required for multiple rounds of transport, however, as NTP hydrolysis is

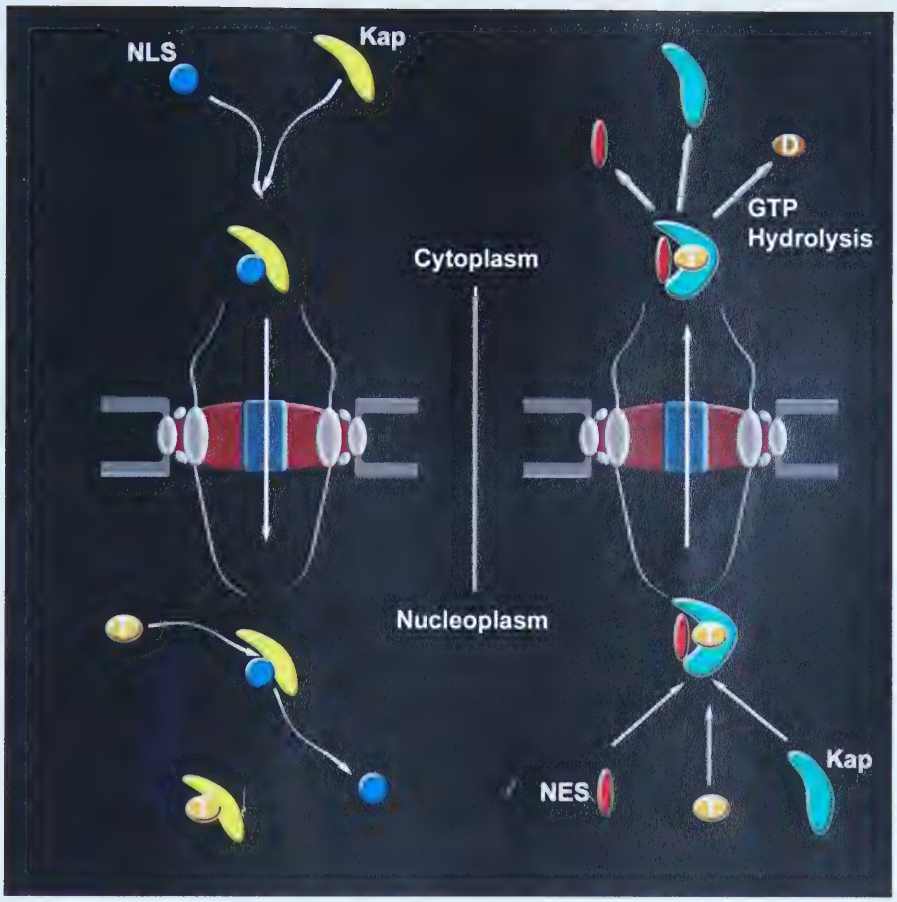

Figure 1-3. Karyopherin-mediated nuclear transport.

An import kap (yellow) recognizes an NLS-containing protein (blue) in the cytoplasm and docks it at the NPC for subsequent translocation. The nucleoplasm contains a high concentration of Ran-GTP (orange/T) which binds the kap cargo complex and displaces the NLS. An export kap interacts simultaneously with an NES (red) and Ran-GTP which is dissociated upon GTP hydrolysis in the cytoplasm. Ran-GDP is shown in a darker orange (D).

necessary for the export of karyopherins (Kose et al., 1997; 1999; Schwoebel et al., 2002). Similarly, a given export process requires energy. A stable complex of a kap, NES and Ran-GTP occurs in the nucleus which is subsequently disassembled by the hydrolysis of Ran-GTP by Ran-GAP on the cytoplasmic face of the NPC (Fig. 1-3; Gorlich and Kutay, 1999; Macara, 2001; Weis, 2003; Fried and Kutay, 2003). The molecular mechanisms through which Ran-GTP modulates kap-cargo interactions have been elegantly described by structural analysis (Vetter et al., 1999; Chook and Blobel, 1999; Chook et al., 2002; Matsuura and Stewart, 2004).

1.4.4 Other transport factors

While the majority of nuclear transport is regulated by karyopherins and Ran, there are other soluble transport factors that have dedicated and essential roles in transporting specific molecules. As a case in point, another highly conserved family of transport factors called nuclear exports factors (NXFs) are largely responsible for exporting mRNA (Izaurralde, 2001; Erkmann and Kutay, 2004). These proteins are structurally unrelated to kaps, and their interactions with mRNA (in the form of RNPs) are not governed by Ran. While they may not be related to kaps, NXFs have a structural domain that is similar to another transport factor, Ntf2p. Ntf2p imports Ran-GDP and is responsible for establishing the concentration gradient of Ran across the nuclear envelope (Smith et al., 1998; Ribbeck et al., 1998). Interestingly, both of these protein families interact directly with FG regions of nups suggesting that the mechanisms that they use to translocate through the NPC are similar to those used by kaps (Fribourg et al., 2001; Bayliss et al., 2002).

1.5 Nuclear transport models

Despite our near comprehensive inventory of the nuclear transport machinery, the way in which these components functionally interact to drive nuclear transport is still limited to the initial and final events of the transport process. Specifically, those involving kap-cargo recognition and the eventual dissociation of the complex by Ran-GTP and its co-factors (see Fig. 1-3 for a graphical depiction of nuclear transport). The mechanisms involved in moving kap-cargo complexes through the central channel of the NPC are still ill defined. These mechanisms will rely heavily on the nature of the biochemical interactions between kaps and nups, and the biophysical properties of the NPC as a whole. A number of models have been proposed to shed light on this process.

1.5.1 The Selective Phase

One model, proposed by Ribbeck and Gorlich (2001;2002) envisions that the central channel is a meshwork of hydrophobic interactions mediated by the FG regions of nups. In this fashion, the central channel is proposed to form a hydrophobic 'selective phase' that would repel large hydrophilic molecules while selectively 'solvating' molecules that can interact with them. Kaps have the ability to interact with the FGs and are therefore thought to locally disrupt this matrix, and partition into the channel. The diffusion of smaller molecules is thought to occur through discontinuities within the matrix. This model is supported by evidence that suggests that transport through the NPC appears to be non-directional and independent of energy (Kose et al., 1997; Nakielny and Dreyfuss, 1998; Ribbeck et al., 1998;1999; Schwoebel et al., 1998; Englmeier et al., 1999; Nachury and Weis, 1999). In addition, biochemical evidence

suggests that a hydrophobic affinity column is selective for kaps over the majority of other cytoplasmic proteins. The NPC permeability barrier can also be disrupted by using low concentrations of alcohols, further suggesting that the channel may be formed from hydrophobic interactions (Ribbeck and Gorlich, 2002). This model, however, does not adequately explain how kaps move through the central channel once they have partitioned into it (Bickel and Bruinsma, 2002).

1.5.2 The Virtual Gate

The Virtual Gate model predicts that the vast majority of molecules are excluded from the NPC channel due to their inherent entropy (Rout et al., 2000; 2003). In this model, the FG-nups are considered to be flexible disorganized proteins that occlude the openings to the central transporter, and brush away molecules that cannot interact with them. In this fashion they further increase the entropic barrier of the NPC. Evidence supporting this model is derived from the observation that the majority of the FG-nups concentrate on both the cytoplasmic and nucleoplasmic sides of the NPC (Rout et al., 2000). Therefore, by extending into these compartments, the tentacle-like FG-nups can recruit and concentrate kap-cargo complexes at the entrances to the NPC. In this fashion, the kaps pay the energy cost to overcome the entropic barrier imposed by the NPC. Once within the channel, this model proposes that kap-cargo complexes move through the NPC by Brownian motion (Rout et al., 2000; Rout et al., 2003).

1.5.3 The Affinity Gradient

In order to achieve rates of import sufficient to accommodate the huge flow of molecules across the NE, both the selective phase and virtual gate models rely on evidence that suggests that the kap-FG interaction is weak (Bayliss et al., 1999; Bayliss et al., 2002; Ribbeck and Gorlich, 2001). Differences in affinity between kaps and FG-nups have been measured, however, and there is a tendency for the affinity of kaps for certain FG-nups to increase from the cytoplasmic filaments to the nuclear basket (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). These observations have been incorporated into an 'affinity gradient' model of nuclear transport, whereby kaps are pulled through the pore by an ever increasing affinity for nups along their route.

1.5.4 Model synopsis

A number of other nuclear transport models have been proposed. The "oily spaghetti" model is similar to the selective phase, but envisions that the central channel is a tube lined with FG-nups, that surround an open diffusion channel (Macara, 2001). Other models predict that molecular rearrangements between certain nups open or close a gate to the central channel (Shulga and Goldfarb, 2003). In the aggregate, these models provide a basic framework for understanding transport, and represent broad strokes in what is likely a finely detailed landscape. For example, the models discussed above do not adequately incorporate a growing body of evidence that suggests that kaps do not all travel similar routes through the NPC. There are clearly specific binding sites, some of which are of high affinity, that have been proposed to play roles in regulating specific transport pathways (see Section 1.6.2). Furthermore, these models place tremendous

emphasis on the FG-repeats as the main effectors of nuclear transport, which may in fact be an overstatement. As a case in point, a recent study evaluates the importance of individual FG-repeats in nuclear transport by systematically deleting FG regions of nups (Strawn et al., 2004). Amazingly, half of the total mass of FGs could be simultaneously deleted without dramatically affecting cell viability. Furthermore, while transport defects were observed, they were linked to specific transport pathways and did not represent a general inhibition of import and export processes. These data are therefore consistent with a model of nuclear transport that is more elaborate than those described above, supporting the idea that there are distinct transport routes through the NPC that are followed by specific karyopherins. They also underscore the involvement of non-FG binding sites in key transport roles.

1.6 Nuclear transport regulation

As a blueprint has emerged for the basic mechanisms of nuclear transport, so has the idea that specific transport pathways can be regulated to orchestrate changes in nuclear physiology including gene transcription, DNA replication, and chromosome segregation. The mechanisms governing this regulation can be broadly grouped into two categories: mechanisms that directly affect kap binding to cargo and those that encompass regulation of the kap-nup interaction. Here, we will highlight some of the mechanisms governing the kap-cargo interaction as they have been extensively reviewed elsewhere (Kaffman and O'Shea, 1999) and will focus on the regulation of transport by the NPC.

1.6.1 Regulating the Kap-cargo interaction

Certainly the most well studied form of transport regulation relies on the modification or masking of an NLS or NES from its cognate kap, to prevent its import or export. Modifications such as phosphorylation and acetylation have been shown to either enhance or inhibit the binding of cargos to karyopherins (Kaffman and O'Shea, 1999; Madison et al., 2002). There is also recent evidence that implicates methylation as a key regulator of the import of certain NLSs (Smith et al., 2004). As an example, the distribution of the yeast transcription factor Pho4p is determined by binding to either the import kap Kap121p or the export kap Kap142p/Msn5p (Kaffman et al., 1998a; Kaffman et al., 1998b). Under phosphate-rich conditions Pho4p is phosphorylated, which results in the simultaneous inhibition of its interaction with Kap121p (import inhibition) and stimulation of its interaction with Kap142p/Msn5p (increased export), the end result being the exclusion of Pho4p from the nucleoplasm. Under conditions of phosphate-starvation, Pho4p becomes dephosphorylated which allows it to interact with Kap121p and prevents its interaction with Kap142p/Msn5p, driving the accumulation of Pho4p in the nucleus and subsequent transcriptional activation. A reciprocal effect is observed with cyclin B1, which accumulates in the nucleus at the beginning of mitosis due to a block in export caused by its phosphorylation (Yang et al., 1998).

Several examples also exist where protein-protein associations between members of a complex can be altered to expose or hide a transport signal from a kap. p53, a well studied transcription factor linked to tumor suppression, dynamically shuttles between the nucleus and cytoplasm. When cells are stressed, p53 is tetramerized which enhances its ability to bind DNA and activate transcription. Interestingly, the tetramerization domain

overlaps a leucine-rich NES that is recognized by the export kap Crm1p. Tetramerization of p53 thus hides the NES from Crm1p and effectively traps p53 in the nucleus, allowing it to continually promote transcription (Stommel et al., 1999).

1.6.2 Regulation of transport by the NPC

In addition to mechanisms that modulate interactions between kaps and their cargos, there is accumulated evidence supporting a role for the NPC in changing levels of nuclear transport. One can envision mechanisms that both globally alter the permeability of the NPC as well as those that affect the transport of specific karyopherins and their cargos. For example, permeability changes driven by molecular rearrangements of the NPC could occur in response to changes in cellular physiology. In support of this idea, evidence in mouse BALB/c 3T3 cells suggests that the size of the NPC channel can significantly change. When comparing nuclear transport between proliferating and quiescent cells, Feldherr and Akin (1993) observed that the apparent size of the NPC translocation channel was larger in cells that were actively growing. They also detected changes in NPC permeability during the cell cycle, reinforcing the idea that the NPC can gear its transport capability to elicit cellular functions (Feldherr and Akin, 1994). The molecular mechanisms causing these changes, however, are not yet known.

1.6.2.1 Different NPCs

Along similar lines, it is hypothesized that cells may regulate transport by altering the complement of nucleoporins in NPCs. This has been observed at both the level of individual NPCs as well as in the tissue-specific expression of certain nup genes. Mlp1p,

for example, is absent from NPCs surrounding the nucleolus in yeast. The functionality of this unique distribution is not yet clear, but is proposed to link transcription with mRNA export by promoting the nuclear retention of unspliced mRNAs in regions associated with chromatin (Galy et al., 2004). Importantly, these data raise the exciting possibility that cells can regulate the function of individual pores and, perhaps, their ability to transport specific cargos.

This idea may also be relevant to the unique tissue-expression patterns of certain nup genes which hint that tissues may regulate nuclear transport to serve specific needs. Recent work has detailed differences in the expression of gp210 and Nup50 in different tissues (Olsson et al., 2004; Smitherman et al., 2000). In addition, the *Drosophila* homolog of Nup88, *members only* (*mbo*), is differentially expressed during larval development in specific cell types (Uv et al., 2000). Interestingly, *mbo* mutants exhibit developmental defects that could be linked to tissue-specific import defects. These data suggest that tissues that lack *mbo* expression have NPCs that are structurally and functionally distinct and that they play key roles in larval development. Importantly, *mbo* mutants were also shown to have nuclear import defects for only certain NLS-bearing proteins, suggesting that *mbo* gene product plays a role in controlling specific transport pathways (Uv et al., 2000).

1.6.2.2 Pathways through the pore

The observation that there are changes in specific transport pathways in the absence of individual nucleoporins suggests that certain nups can potentially directly influence the transport of specific subsets of cargos. This idea is supported by numerous

studies in both yeast and vertebrates. A few interesting examples come from evaluating the effects of viral infection on nuclear transport. During poliovirus infection two nups, Nup153 and p62 are selectively degraded by viral proteases which correlates with the inhibition of distinct nuclear import pathways (Gustin and Sarnow, 2001). Similar effects are observed during a vesicular stomatitis virus infection (Her et al., 1997; Peterson et al., 2000). Although in this case, transport attenuation is thought to be a direct consequence of the inhibitory effects of the viral M-protein through an interaction with Nup98 (von Kobbe et al., 2000).

The effects on nuclear transport observed upon viral infection are supported by a number of studies evaluating the role that Nup98 and Nup153 play in regulating distinct nuclear transport pathways. Injection of anti-Nup98 antibodies into *Xenopus* oocytes results in the abrogation of snRNA, mRNA and rRNA export but does not affect the export of tRNA (Powers et al., 1997). Similar experiments using antibodies directed against Nup153 also have no effect on tRNA export, but clearly inhibit the export of snRNA, mRNA and 5sRNA (Ullman et al., 1999). Perturbations of nuclear import pathways have also been observed. The immunodepletion of Nup153 from *Xenopus* extracts results in the disruption of Kap-β1/Kap-α mediated import without effecting the Kap-β2 transport pathway (Walther et al., 2001). Furthermore, the overexpression of dominant negative truncations of Nup153 can have specific effects on both the Kap-β1 and Kap-β2 import pathways (Shah and Forbes, 1998).

1.6.2.3 Specific binding sites

One logical explanation of these results is that there are unique pathways traveled by karyopherins as they move through the NPC that involve their binding to specific sites on nups. In support of this idea, Nup53p has been shown to interact specifically with only one kap, Kap121p (Marelli et al., 1998). More of these specific interactions are exemplified by *in vitro* pulldown experiments using GST-tagged nups (Allen et al., 2001). These experiments revealed that there were preferential interactions between certain kaps and nups which provides support for the existence of specific kap binding sites. Furthermore, specific binding sites for Kap-β2 have been mapped on both Nup153 and Nup98 (Nakielny et al., 1999; Fontoura et al., 2000; Shah et al., 1998). In yeast, a Kap95p-specific binding site on Nup1p, and a Kap60p binding site on Nup2p have also been uncovered (Pyhtila and Rexach, 2003: Gilchrist and Rexach, 2003; Booth et al., 1999; Hood et al., 2000; Solsbacher et al., 2000; Matsuura et al., 2003).

A common feature of the binding sites that have been mapped is that they are devoid of FGs and often act as NLSs. Furthermore, in cases where it has been measured, they have affinities for kaps that are much stronger than the Kap-FG-repeat interaction (Pyhtila and Rexach, 2003; Matsuura et al., 2003; Ribbeck and Gorlich, 2001). Perhaps most significantly, there is evidence linking these sites to the regulation of distinct nuclear import pathways (Gilchrist and Rexach, 2003; Pyhtila and Rexach, 2003; Matsuura et al., 2003). The deletion of the Kap95p binding site in Nup1p, for example, lowers its binding affinity for Kap95p 450-fold and has specific effects on Kap95p/Kap60p mediated import (Pyhtila and Rexach, 2003). Similarly, mutating key

amino acid residues in the Kap60p binding site on Nup2p affects the efficiency of NLS import (Gilchrist and Rexach, 2003; Matsuura et al., 2003).

In many cases, nups containing these binding sites appear to cluster on the nuclear basket (Rout et al., 2000; Cronshaw et al., 2002, Suntharalingam and Wente, 2003). The significance of this distribution may reflect a role for these sites in promoting the dissociation of cargo from karyopherins, and thus an aid in the termination step of specific import reactions (Fontoura et al., 2000; Gilchrist and Rexach, 2003; Matsuura et al., 2003). This idea has been developed in detail with respect to the Kap60p/Nup2p interaction, and has culminated with the elucidation of the crystal structure of Kap60p in complex with the high affinity Nup2p binding site (Matsuura et al., 2003). This structure revealed that the Nup2p peptide bound to a region of Kap60p that partially overlapped the NLS-binding groove. Since Kap60p binds Nup2p with a higher affinity than NLSs, this interaction is thought to displace the NLS. This observation has been independently supported biochemically by other groups (Solsbacher et al., 2000; Gilchrist and Rexach, 2003).

1.7 Post translational modification of the NPC

As alluded to above, the existence of specific transport pathways likely allows cells to independently control the transport of distinct classes of molecules. The examples used, however, generally consider mutants in specific nups that resulted in changes in a particular transport pathway. Clearly, in order for cells to actively regulate these pathways requires that they can directly alter individual nups or direct specific

changes in NPC architecture, or nup interactions. One logical way in which cells could perform these functions is to use post-translational modifications.

There is abundant evidence that the NPC is a target a number of post-translational modifications including glycosylation, phosphorylation and sumoylation (Miller et al., 1999; Li et al., 2004). So far, glycosylation appears to be unique to higher eukaryotes, as it has not been detected at the yeast NPC (Davis, 1995). In addition, preventing nup glycosylation does not appear to have a direct effect on nuclear transport or NPC architecture (Miller and Hanover, 1994). While a conclusive function of nup glycosylation is still lacking, glycosylated amino acid residues are often identical to those that are targets of kinases. It has been proposed, therefore, that glycosylation masks phosphorylation sites to temporally regulate nup phosphorylation, which is thought to have more potent consequences on NPC function (Hart et al., 1997).

1.7.1 The phosphorylation of nucleoporins

In agreement with the idea that the phosphorylation of the NPC affects its function, there is accumulated evidence that supports the involvement of nup phosphorylation in regulating nuclear transport. For example, a number of studies have evaluated the effect of kinase and phosphatase inhibitors on specific import and export pathways. In addition, certain kinases have been incubated in *in vitro* import assays to drive the phosphorylation of transport components. These studies provide evidence that the phosphorylation of the transport machinery, likely the NPC itself, may both up and down regulate nuclear transport (Feldherr and Akin, 1995; Mishra and Parnaik, 1995; Kehlenbach and Gerace 2000). However, the nups that are the targets of these

phosphorylation events, and the transport mechanisms that are influenced are completely unknown.

There a number of vertebrate nups that have been observed to be phosphorylated including Nup153, Nup214 and Nup358 (Macaulay et al., 1995, Favreau et al., 1996). These nups appear to be phosphorylated throughout the cell cycle, but the level of their phosphorylation increases in mitosis concomitantly with the phosphorylation of other nups including gp210 and Nup53 (Favreau et al., 1996; Stukenberg et al., 1997; L.A. Hawryluk-Gara and R.W. Wozniak, unpublished). It has been proposed that the phosphorylation of nups that occurs during mitosis plays an important role in breaking nup-nup interactions and thereby contributes to the mechanisms governing NPC disassembly and reassembly (Macaulay et al., 1995; Favreau et al., 1996). While there are no biochemical data to confirm this idea, evidence from the fungus *Neurospora crassa* suggests that the phosphorylation of the NPC (perhaps Nup98 and Gle2) may play a role in the dissociation of at least 5 nups from the NPC, supporting this hypothesis. These events occur concomitantly with the opening of the diffusion channel across the NPC which allows the diffusion of tubulin and other mitotic regulators such as Cdk1/Cyclin B into the nucleus (De Souza et al., 2004). Tubulin and Cdc2/CyclinB import are critical for progression into mitosis and the formation of the mitotic spindle (Wu et al., 1998; Ovechkina et al., 2002). These results may also provide evidence for the existence of a key evolutionary intermediate between organisms with a closed and an open mitosis and suggests that mechanisms that cause the dissociation of nups from the *N. crassa* NPC are similar to those causing the more dramatic disassembly and reassembly of the NE that occur in vertebrates.

1.8 NPC assembly

Principally, research into the mechanisms governing NPC assembly have focused on defining a temporal order of nup incorporation into a growing NE, and nascent NPC (Bodoor et al., 1999). This type of analysis has also led to the identification of a number of assembly intermediates which have been visualized on the surface of NEs (Goldberg et al., 1997; Gant et al., 1998; Kiseleva et al., 2001), but they have been limited by their lack of mechanistic insight into this process. NPC assembly occurs throughout the cell cycle in both yeast and vertebrates (Maul et al., 1971; 1972; Winey et al., 1997) and it has been traditionally thought that there are mechanisms that drive the assembly of NPCs during NE formation at the end of mitosis, and, those that function to insert NPCs into intact nuclear envelopes. The existence of the latter mechanisms have particular relevance in yeast, where the NPCs do not disassemble and the NE remains intact throughout the cell cycle. It remains a possibility, however, that NPC assembly occurs by similar mechanisms in both cases. This idea is prompted by recent evidence whereby the components required for NPC assembly appear to be similar in both yeast and in vertebrates. For example, Ran-GTP provides a spatial and temporal cue for nuclear assembly reactions during mitosis in vertebrates. Through an interaction with chromatin-associated Ran-GEF, a local concentration of Ran-GTP is created surrounding the DNA (Hinkle et al., 2002; Bilbao-Cortes et al., 2002). Here, Ran-GTP is important at numerous steps of NPC assembly. It is required to recruit nups to the chromatin surface, but also functions in vesicular fusion events, and in later steps, the insertion of NPCs into NEs (Hetzer et al., 2000; Zhang and Clarke, 2000; Walther et al., 2003). A similar

requirement for Ran and its co-factors in NPC assembly has been observed in yeast (Demeter et al., 1995; Matynia et al.,1996; Ryan et al., 2003). Mutations in components of the Ran pathway have dramatic effects on the incorporation of newly synthesized nups into NPCs (Ryan et al., 2003). The mislocalized nups appear in small vesicles alongside of significant NE abnormalities, suggesting that Ran may also function in vesicular fusion events, and NE maintenance. It is not clear, mechanistically, how Ran influences these processes. One intriguing result observed in *Xenopus in vitro* assembly reactions suggests that Ran-GTP functions to release Kap-β1 from nups, which allows them to form higher order interactions and thus build the NPC (Walther et al., 2003).

1.9 Thesis focus

This thesis explores the functional relationships between a subcomplex of the NPC that contains the nups, Nup53p, Nup59p, Nup170p and Nic96p as well as the kap, Kap121p. By dissecting the interactions between Nup53p and these components we reveal mechanisms supporting (A) that kaps can function in NPC assembly (B) that Kap121p-mediated import is inhibited in mitosis by molecular rearrangements within this complex and (C) that the phosphorylation of Nup53p is controlled by two mitotic kinases.

Chapter II: *Experimental Procedures*

2.1 Yeast strains and media

All strains were grown at 30°C unless otherwise indicated in YPD (1% yeast extract, 2% bactopeptone, and 2% glucose), YP-Gal (1% yeast extract, 2% bactopeptone, and 2% galactose) or in synthetic media (SM) supplemented with the necessary nutrients containing 2% glucose (Sherman et al., 1983). Yeast transformations were performed as described by Delorme (1989) and tetrad dissections as in Sherman (1983). See Table I for a list of strains used for this thesis. CPL14, CPL35 and CPL75 are segregants from the cross of CPL32 with lte1Δ, ctf18Δ and bim1Δ, respectively. CPL69 is a segregant derived from the mating of CPL6 and CPL7. To produce CPL32, a *KANR* gene cassette was amplified by a PCR using chromosomal DNA isolated from nup53Δ as a template. Oligonucleotide primers were designed to anneal to the following nucleotides of the *NUP53* gene locus: 1828-1848 and the complement of -400 to -380, where 1 is the A of the *NUP53* initiation codon. The resulting PCR product was transformed into BY4741 and the knockout of *NUP53* in the resulting strain was confirmed by Western blotting with anti-Nup53p antibodies. To produce yeast strains CPL37 and CPL66, oligonucleotide primers were designed to amplify by a PCR a region of p315-NUP53 and p315-nup53-2A. The "sense" primer corresponded to nucleotides -400 to -380 upstream of the *NUP53* initiation codon (1 is the A of the *NUP53* initiation codon), and the "antisense" primer annealed to a region 100 bp downstream of the *LEU2* gene. A 60 bp 5' overhang was designed on the antisense primer to allow for homologous recombination with a region in the 3'UTR (100 bp from *NUP53* termination codon) at the *NUP53* gene locus. The resulting PCR products were transformed into CPL32 and colonies were selected for growth on SM lacking leucine. The integration of *NUP53* and

nup53-2A at the *NUP53* gene locus was suggested by the lack of growth on media containing G418 (the resistance to G418 is conferred by the *KANR* gene product). The production and stability of Nup53p and nup53-2A were assessed by Western blotting (Fig. 5-2B).

p315-NUP53 and p315-nup53-2A were transformed into NP53NUP170-11, colonies were selected and subsequently plated onto media containing 5-Fluoro-orotic acid to force the loss of p316A-NUP170, and produce CPL71 and CPL72. p315-NUP53 and p315-nup53-2A were also transformed into CPL32 to produce CPL73 and CPL74. pYNP53, pYΔ405-430 and pY1-448 were transformed into NP53-A2 to produce NP53-FL53, NP53-Δ405-430 and NP53-1-448 respectively.

Genomic integrations of the *GFP* and *YFP* ORFs were performed in a diploid DF5 strain or the strain KP121-41 to produce NP53GFP, NP59GFP, NP53GFP/KP121-41, NP59GFP/KP121-41, and NP170YFP. Similar integrations of *GFP*$^+$ and *RFP* ORFs were performed in BY4742 and BY4741 to produce CPL6 and CPL7, respectively. Briefly, oligonucleotides were designed to anneal to identical sequences within pGFP/HIS5, pGFP$^+$/HIS5, pYFP/URA4 and pRFP/HIS5 (described in Aitchison et al., 1995b), to allow for the amplification of the *GFP*, *GFP*$^+$, *YFP*, *RFP* ORFs, respectively. The "sense" and "antisense" oligonucleotides were designed with 5' overhangs which anneal to a region complementary to a 60 bp sequence directly upstream, or to a 60 bp sequence 100 nucleotides downstream of the endogenous gene termination codon, respectively. A similar strategy was used to produce the following strains containing YFP-tagged constructs using the plasmids pRNP53-YFP, pRΔ405-430-YFP, pRNP53-cNLS-YFP and pRNP53-mNLS-YFP as PCR templates: NP53YFP, Δ405-430-YFP,

NP53-cNLS-YFP and NP53-mNLS-YFP. In these cases, the "sense" oligonucleotide was designed to anneal to the complement of nucleotides 1000-1020 (where 1 is the A of the *NUP53* initiation codon) of *NUP53* and the *nup53* mutants, and the "anti sense" oligonucleotide was designed to anneal 100 bp downstream of the *LEU2* gene in the plasmid templates.

The synthesis of each of the GFP, GFP$^+$ and YFP fusions was confirmed by immunoblotting total cell extracts using either anti-Nup53p, anti-Nup59p (Marelli et al., 1998) or anti-GFP antibodies (gift of Mike Rout, The Rockefeller University, USA). The synthesis of Spc42-RFP was confirmed by microscopy.

Table 2-1. Yeast Strains

Name	Genotype	Reference
DF5	*MAT a ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801*	Marelli et al., 1998
W303	*MAT a ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Marelli et al., 1998
YPH499	*MAT a ura3-52 lys2-801 ade2-101 trp-63 his3-200 leu2-1*	Sikorski and Heiter, 1989
BY4741	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Giaever et al., 2000
BY4742	*MAT α his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Giaever et al., 2000
yck1Δ	BY4742 except *yck1Δ::KANR*	Giaever et al., 2000
yck2Δ	BY4742 except *yck2Δ ::KANR*	Giaever et al., 2000
yck3Δ	BY4742 except *yck3Δ ::KANR*	Giaever et al., 2000
hal5Δ	BY4742 except *hal5Δ ::KANR*	Giaever et al., 2000
fus3Δ	BY4742 except *fus3Δ::KANR*	Giaever et al., 2000
tpk1Δ	BY4742 except *tpk1Δ::KANR*	Giaever et al., 2000
ptk2Δ	BY4742 except *ptk2Δ ::KANR*	Giaever et al., 2000
ste20Δ	BY4742 except *ste20Δ::KANR*	Giaever et al., 2000
nup53Δ	BY4742 except *nup53Δ::KANR*	Giaever et al., 2000
bub2Δ	BY4742 except *bub2Δ::KANR*	Giaever et al., 2000
msn5Δ	BY4742 except *msn5Δ::KANR*	Giaever et al., 2000

Name	Genotype	Reference
cdc26Δ	BY4742 except *cdc26Δ::KANR*	Giaever et al., 2000
lte1Δ	BY4742 except *lte1Δ::KANR*	Giaever et al., 2000
ctf18Δ	BY4742 except *ctf18Δ::KANR*	Giaever et al., 2000
bim1Δ	BY4742 except *bim1Δ::KANR*	Giaever et al., 2000
NP53-A2	DF5 except *nup53Δ::HIS3*	Marelli et al., 1998
KP121-41	DF5 except *kap121Δ::LEU2,* pkap121-41	Leslie et al., 2002
NP53GFP	DF5 except *NUP53-GFP::HIS5*	This study
NP59GFP	DF5 except *NUP59-GFP::HIS5*	This study
NP53GFP/KP121-41	KP121-41 except *NUP53-GFP::HIS5*	This study
NP59GFP/KP121-41	KP121-41 except *NUP59-GFP::HIS5*	This study
NP53YFP	DF5 except *NUP53-YFP::URA3*	This study
Δ405-430-YFP	DF5 except *405-430-YFP::URA3*	This study
NP53-cNLS-YFP	DF5 except *NUP53-cNLS-YFP::URA3*	This study
NP53-mNLS-YFP	DF5 except *NUP53-mutcNLS-YFP::URA3*	This study
NP170YFP	DF5 except *NUP170-YFP::LEU1*	This study
NP53NP170-11	*MAT a ade2 ura3 his3 trp1 leu2 nup53Δ::HIS3 nup170Δ::HIS3* pRS316A-NUP170 (*URA3*)	Marelli et al., 1998
CPL6	BY4742 except *SPC42-RFP::HIS5*	This study
CPL7	BY4741 except *HRR25-GFP::HIS5*	This study
CPL14	BY4741 except *nup53Δ::HIS3 lte1Δ::KANR*	This study
CPL32	BY4741 except *nup53Δ::KANR*	This study
CPL35	BY4741 except *nup53Δ::HIS3 ctf18Δ::KANR*	This study
CPL37	CPL32 except *nup53Δ::kanr::NUP53::LEU2*	This study
CPL61	YPH499 except *GAL1::MPS1::URA3*	Scott et al., 2005
CPL66	CPL32 except *nup53Δ::kanr::nup53-2A::LEU2*	This study
CPL69	BY4741 except *SPC42-RFP::HIS5 HRR25-GFP::HIS5*	This study
CPL71	NP53NP170-11 except p315-NUP53	This study
CPL72	NP53NP170-11 except p315-nup53-2A	This study
CPL73	CPL32 except p315-NUP53	This study
CPL74	CPL32 except p315-nup53-2A	This study
CPL75	BY4741 except *nup53Δ::HIS3 bim1Δ::KANR*	This study
KKY387	YPH499 except *hrr25Δ::loxP-kanMX-loxP* , pKK204	Kafadar et al., 2003
NP53-FL53	DF5 except *nup53Δ::HIS3*, pYEX-53	This study

Name	Genotype	Reference
NP53-Δ405-430	DF5 except *nup53Δ::HIS3*, pYEX-Δ405-430	This study
NP53-1-488	DF5 except *nup53Δ::HIS3*, pYEX-1-488	This study
NP53/NP59-2.1	*nup53Δ::HIS3 nup59Δ::HIS3*	Marelli et al., 1998
NP170pA	DF5 except *NUP170-pA::HIS3::URA3*	Aitchison et al., 1995b
KP121pA	DF5 except *KAP121-pA::HIS3::URA3*	Rout et al., 1997
NP170-11.1	DF5 except *nup170Δ::HIS3*	Aitchison et al., 1995b
NP120-25-3	DF5 except *nup120Δ::LEU2*	Aitchison et al., 1995a
nic96-1	W303 except *nic96Δ::HIS3,* pnic96-1 (*TRP1*)	Grandi et al., 1995
NP53/nic96-1	*nup53Δ::HIS3 nic96Δ::HIS3* pnic96-1	This study
NP53/NP120	DF5 except *nup53Δ::HIS3 nup120 Δ::LEU2*	This study
KP121pA/NP170YFP	DF5 except *KAP121-pA::HIS3::URA3 NUP170-YFP::LEU1*	This study
NP53pA	DF5 except *NUP53-pA::HIS3*	Marelli et al., 1998
cdc15-2B	*cdc15-2::LEU2*	Marelli et al., 1998
KP121-34	DF5 except *kap121Δ::LEU2*, pkap121-34	Leslie et al., 2002
NIC96-pA	DF5 except *NIC96-pA::HIS5*	Rout et al., 2000

2.2 Plasmids

The following plasmids were used for this thesis: pGFP/HIS5 (Dilworth et al., 2001), pGFP^{+}/HIS5, pRFP/HIS5 (Scott et al., 2005), pYFP/URA4 (gift from Rick Rachubinski, University of Alberta, Canada), pPHO4-NLS-GFP (EBO836)(Kaffman et al., 1998; provided by E. O'Shea, University of California, San Francisco, San Francisco, CA), pSTE12-NLS-GFP (pSte12p (aa 494-688)-GFP) (Leslie et al., 2002), pcNLS-GFP (Stade et al., 1997), pKAP121-GFP (pPS1069) (Seedorf and Silver, 1997; provided by P. Silver, The Dana Farber Cancer Institute, Boston, MA), pRG-NLS-GFP (Lee and Aitchison, 1999), pRPL25-NLS-GFP (provided by M. Rout, Rockefeller University, New

York, NY), pYEX-BX (Clontech), pYNP53, pY1-448, pGEX-KAP121 (Marelli et al., 2001), pGFP-TUB1 (provided by N. Adames, University of Alberta, Edmonton, AB; Adames et al., 2001) and pPDS1-HA (provided by M. Basrai, National Institutes of Health, Bethesda, MA).

All of the following plasmids have inserts of PCR products amplified with the Expand High Fidelity PCR System (Roche) using oligonucleotide primers with *Bam*HI linkers. The PCR products were inserted into the *Bam*HI site of the indicated plasmid. The following regions of *NUP53*, where 1 is the A of the initiation codon, were inserted into pGEX-4T-1 (Amersham Biosciences): pGNUP53 (1-1428), pG203-475 (607-1428), pG374-475 (1120-1428), pG401-448 (1201-1344). The following regions of *NUP53* containing a 5' *Bam*HI site and a 3' blunt end were inserted into pGEX-3X (Amersham Biosciences) cut with *Bam*HI and a filled in *Eco*RI site. pG1-375 (1-1126), pG1-297 (1-892), pG1-201 (1-604). *NUP53* was excised from pGNUP53 by *Bam*HI digestion and subcloned into the *Bam*HI site of pGEX-6P-1 to produce pG6PI-NUP53.

All of the following regions of *NUP53* containing a 5' *Bam*HI site were subcloned into the *Bam*HI site in pGFP-C-fus (Niedenthal et al., 1996): pNP53-GFP (–8 to 1428), p203-475-GFP (-3 to 3 and 607-1428), p374-475-GFP (–3 to 3 and 1120-1428), p456-475-GFP (–3 to 3 and 1366-1428), p1-448-GFP (–8 to 1343), p1-375-GFP (–8 to 1119), p1-206-GFP (–8 to 617), pKBD-GFP (–3 to 3 and 1201-1343).

The deletion constructs of the *NUP53* ORF lacking the Kap121p binding domain and containing an insertion of a cNLS and a mutant cNLS were made as follows. Antisense and sense oligonucleotides corresponding to nucleotides +1199-1215 and +1299-1318, respectively, of *NUP53* were designed with 5' *Bgl*II linkers. These primers

were used in a PCR to synthesize a linear copy of the pNP53-GFP plasmid lacking the coding region for amino acid residues 405-430. This cDNA was digested with *Bgl*II and ligated to produce the pΔ405-430-GFP plasmid. The plasmids pNP53-cNLS-GFP and pNP53-mNLS-GFP were constructed by inserting the coding region of the SV40 large T antigen NLS (cNLS) or a mutant of the cNLS (the "sense" oligonucleotide primers were synthesized as 5' GATCTCATAGTACTCCACCAAAAAAAAAAAGAAAAGTTG AAGATCCAAAA and the mutant as 5'GATCTCATAGTACTCCACCAAAAACAA AAAGAAAAGTTGAAGATCCAAAA) into the *Bgl*II site of pΔ405-430-GFP.

The plasmids pRNP53-YFP, pRΔ405-430-YFP, pRNP53-cNLS-YFP and pRNP53-mNLS-YFP were constructed as follows. The *EYFP* ORF was amplified by a PCR from pEYFP (Clontech Laboratories Inc.) with oligonucleotide primers containing *Bam*HI and *Not*I linkers. The resulting PCR product was directionally inserted into the *Bam*HI and *Not*I sites of pRS306 (*URA3,* Sikorski and Hieter., 1989) to produce pRYFP. The ORFs coding for *NUP53*, *Δ405-430*, *NUP53-cNLS* and *NUP53-mut-cNLS* were excised by *Bam*HI digestion from pNUP53-GFP, pΔ405-430-GFP, pNUP53-cNLS-GFP and pNUP53-mNLS-GFP and subsequently ligated into the *Bam*HI site of pRYFP producing pRNP53-YFP, pRΔ405-430-YFP, pRNP53-cNLS-YFP, and pRNP53-mNLS-YFP. *Δ405-430* was subcloned into pYEX-BX using *Bam*HI, from pΔ405-430-GFP to produce pYΔ405-430.

The *PHO4* ORF was amplified by a PCR from yeast genomic DNA using oligonucleotide primers with *Bam*HI linkers. The resulting PCR product was inserted into the *Bam*HI site of pGEX-6P-1 (Amersham Biosciences) to construct pGPHO4. To

construct pPHO4-NLS*-GFP, the region encoding the NLS in pPHO4-NLS-GFP was excised by a *Bgl*II, *Eco*RI digestion and replaced by a region encoding a mutant of the Pho4-NLS, Pho4-NLS*. To produce *PHO4-NLS**, two complementary oligonucleotides were designed to change the codons in *PHO4-NLS* that encode serine and threonine amino acid residues to encode alanine residues. The "sense" oligonucleotide was synthesized as 5'-GATCTGCCGCTAACAAAGTCGCCAAAAA CAAGGCCAATGCCGCCCCGTATTTGAACAAGCGCAGAGGTAAACCCGGGCCG GATGCCG.

pKK196 (pGEX-4T-3-HRR25) was a generous gift from M. Cyert (Kafadar et al., 2003), and so were pAM4-1 (*HA-HRR25/2μ/TRP1*) and pAM8-2 (*HA-hrr25-K38A/2μ /TRP1*) which were provided by R. Schaffrath (Mehlgarten and Schaffrath, 2003; Murakami et al., 1999). The ORFs encoding *HRR25* and *hrr25-K38A* were excised from pKK196 and pAM8-2 respectively using *Bam*HI and subcloned into the *Bam*HI site of pGEX-6P1 (Amersham Biosciences) to produce pGST-HRR25 and pGST-hrr25-K38A.

p315-NUP53 was constructed in the following steps: the promoter region of *NUP53* was amplified by a PCR using a "sense" oligonucleotide primer designed with an *Xho*I linker to correspond to nucleotides -500 to -480 of the *NUP53* gene locus (1 is the A of the *NUP53* initiation codon), and an "antisense" primer designed with a *Bam*HI linker to anneal to nucleotides -24 to -6. The resulting PCR product was digested with *Xho*I and *Bam*HI and subcloned into the *Xho*I and *Bam*HI sites of pRS315 (*CEN/LEU2*, Sikorski and Heiter, 1989) to produce p315-53PR. In a subsequent step, the 3'UTR region from *NUF2* (200 bp including stop codon) was similarly amplified using a "sense" primer with a *Bam*HI linker designed to anneal to the complement of a 20 bp region of

the *NUF2* gene locus that includes the termination codon, and an "antisense" primer with a *Not*I linker designed to anneal to a DNA sequence 200 bp downstream of the *NUF2* termination codon. The resulting PCR product was inserted into the *Bam*HI and *Not*I sites of p315-53PR to produce p315-53PRT. Lastly, the *NUP53* ORF was excised from pNP53-GFP with *Bam*HI and inserted into the *Bam*HI site between the *NUP53* promoter and *NUF2* terminator to construct p315-NUP53.

Using pNP53-GFP as a template, site-directed mutagensis was performed using the QuikChange Site-Directed Mutagensis Kit (Stratagene). Oligonucleotide primers were designed as per the manufacturer's instructions to change the codons encoding serines 101 and 206 in *NUP53* to alanines. The codon encoding serine 101 was changed from AGC to GCC, and serine 206 from TCG to GCG. The codon changes were confirmed by DNA sequencing, and the resulting plasmid was called pnup53-2A-GFP. The *nup53-2A* ORF was excised by *Bam*HI digestion and subcloned into the *Bam*HI site of p315-53PRT to construct p315-nup53-2A.

2.3 Overlay assays

2.3.1 Kap121-pA

Total cell lysates were prepared from *E.coli* cells synthesizing the indicated GST fusion proteins. Proteins from these lysates were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were then blocked with 5% skim milk in Transport Buffer (Table 2-2), and probed with a cytosolic fraction isolated from yeast cells synthesizing a Kap121-pA fusion as previously described (KP121pA/Marelli et al.,

1998). The bound protein A was detected with HRP-conjugated donkey anti-rabbit antibodies and ECL (Amersham Biosciences).

Table 2-2. Buffers

Transport Buffer	50 mM Hepes pH 7.5, 110 mM KOAc, 2 mM $MgCl_2$, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.4 μg/ml pepstatin A
Lysis Buffer A	50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% tween 20
Lysis Buffer B	50 mM Tris-HCl, pH 7.5, 150 mM KOAc, 1 mM MgOAc, 0.1% Nonidet-40, and 10% glycerol
Lysis Buffer C	20 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$, 0.2 mM PMSF, 2 μl/ml leupeptin, 2 μg/ml aprotinin and 0.4 μg/ml pepstatin A
Lysis Buffer D	50mM Tris-HCl, pH 7.5, 0.5M NaCl, 50mM NaF, 5mM EDTA, 1% triton X-100
Kinase Buffer	50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM $CaCl_2$, 2 mM ATP
SDS-PAGE sample buffer	50 mM Tris-Cl pH 6.8, 5% SDS, 0.25% bromophenol blue, 25% glycerol
PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4

2.3.2 Nup170-pA

Overlay assays using Nup170-pA derived from yeast lysates were performed using a similar procedure as in 2.3.1. A yeast strain synthesizing Nup170-pA (NP170pA) was grown in 50 ml of YPD to an OD_{600} of 1.0, harvested by centrifugation, resuspended in in 15 mls of Transport Buffer and lysed by passaging the cells three times through a French Press chamber at 1000Ω. Triton X-100 was then added to a final concentration of 1% and the lysate was incubated on ice for 10 min. The lysate was then clarified by centrifugation at 11,300 x g for 15 min at 4°C. The supernatant fraction was diluted 2x in Transport Buffer and used to probe nitrocellulose membranes as in 2.3.1. For Nup170-

pA overlay experiments performed in the presence of recombinant Kap121p, clarified Nup170-pA lysates were supplemented with purified Kap121p (for purification procedure see 2.4.1) to a final concentration of 0, 50 nM, or 500 nM prior to probing membranes. Bound protein A was detected as in 2.3.1.

Kap121-pA and Nup170-pA (prepared as above) were also used to probe nitrocellulose-immobilized Nup53p which was phoshporylated by M-phase *Xenopus* extracts (gift of E.K. Shibuya).

2.4 Protein purification

2.4.1 Kap121p

A 1 l culture of an *E. coli* BLR 21 strain transformed with pGEX-KAP121 was grown in Terrific Broth (1.2% Tryptone, 2.4% yeast extract, 0.4% glycerol) to an OD_{600} of ~4 at 37°C. IPTG (isopropyl-β-D-galactoside) was added at a final concentration of 0.5 mM to the culture medium to induce the production of GST-Kap121p for 16 h at 25°C. Cells were harvested and resuspended in 50 ml of Lysis Buffer A (Table 2-2) and lysed by passaging the cells three times through a French Press chamber at 1000Ω. The resulting lysate was clarified by centrifugation at 11,300 x g for 15 min at 4°C. The supernatant was then incubated for 1 h at 4°C with 0.5 ml of pre-equilibrated Glutathione (GT)-Sepharose beads. After extensive washing, Kap121p was cleaved from GST by an overnight incubation with 10 U of thrombin (Sigma). Thrombin recognizes a site between GST and Kap121p.

2.4.2 GST-fusions and Nup53p

50 ml cultures of *E. coli* Rosetta* (Lutzmann et al., 2002) strains transformed with plasmids expressing GST-fusion proteins, were grown in 2YT (1.6% Tryptone, 1.0% Yeast Extract, 0.5% NaCl) to an OD_{600} of 1.0. The production of the GST-fusions was induced with the addition of IPTG to a final concentration of 0.5 mM to the growth medium. After 6 h at 25°C the cells were collected by centrifugation, resuspended in 15 mls of Lysis Buffer B and lysed by sonication. The resulting lysate was clarified by centrifugation at 11,300 x g for 15 min at 4°C. The supernatant was then incubated for 1 h at 4°C with 0.1 ml of pre-equilibrated Glutathione (GT)-Sepharose beads. After extensive washing with Lysis Buffer B, the beads were used in binding experiments. To isolate Nup53p, GT-beads containing immobilized GST-Nup53p were incubated for 3 h at 4°C with 10 U of PreScission Protease (Amersham Biosciences) which recognizes a site between Nup53p and GST. The purification of GST, GST-401-448 was performed similarly except that Lysis Buffer A was used.

2.5 *In vitro* binding experiments

2.5.1 For Figure 3-2

Solution binding experiments performed using recombinant Kap121p and the KBD-containing region of Nup53p were conducted as follows. GST and GST-401-448 were purified on GT-Sepharose as described in 2.4.2. 10 µl of beads (~5 µg of GST or GST-401-448) were then incubated with ~10 µg of purified Kap121p (2.4) for 1 h at 4°C. The beads were washed extensively with Lysis Buffer A (Table 2-2). Bound proteins

were eluted with SDS-PAGE sample buffer (Table 2-2), separated by SDS-PAGE and visualized by staining the proteins with BioSafe Coomassie (BioRad).

2.5.2 For Figure 4-5

Solution binding experiments performed using Kap121p, Pho4p and Nup53p were conducted as follows. Purified Kap121p (2.4.1) was incubated with GT-Sepharose bound GST-Pho4p for 1 h at 4°C. After washing, this complex was released from the GT-Sepharose after a 3 h incubation at 4°C with 10 U of PreScission Protease. The PreScission Protease recognizes a site between GST and Pho4p. The Kap121p/Pho4p complex (~10 μg) was then incubated for 1 h at 4°C with 10 μl of GT-Sepharose beads pre-loaded with ~5 μg of GST-Nup53p, or GST-1-375. The beads were washed and eluted with SDS-PAGE sample buffer (Table 2-2). Proteins were separated by SDS-PAGE and visualized by staining the proteins with Bio-Safe Coomassie (BioRad).

2.5.3 For Figure 5-5

For binding experiments with GST-Hrr25p, GST-hrr25-K38A and Nup53p: 60 μL of GT-Sepharose containing ~20 μg of bound GST-Hrr25p or GST-hrr25-K38A were split into two 30 μl aliquots. One aliquot was incubated or mock-incubated with 15 μg of purifed recombinant Nup53p (2.4.2). After a 1 h incubation at 4°C, the beads were washed extensively in Lysis Buffer B, and split into 3, 10 μl aliquots. SDS-sample buffer was added to one and the two remaining aliquots were incubated for 1 h at 30°C in 100 μl of Lysis Buffer B, with or without the addition ATP to a final concentration of 1 mM. Beads were collected and 'release' fractions were precipitated with trichloroacetic acid

(10% v/v) and resolubilized with SDS-sample buffer. Beads were washed an additional two times and SDS-sample buffer was added. Samples of both bound and release fractions were separated by SDS-PAGE and proteins were stained with BioSafe Coomassie (BioRad).

2.6 *In vitro* GST-kinase screen

To identify kinases that phoshporylate Nup53p, 119 of the 122 *S. cerevisiae* kinases were purified as described by Zhu et al. (2000). Briefly, 1 ml cultures of GST-kinase expressing strains were grown overnight in 96-well culture blocks in SM media lacking uracil and containing 3% raffinose as the sole carbon source. At OD_{600} of 0.4-0.6 cells were collected by centrifugation and resuspended in fresh SM–Ura containing 3% raffinose and 3% galactose to induce the synthesis of the GST-kinase. After 3 h, cells were collected by centrifugation and resuspended in 200 µl of Lysis Buffer D. 100 µl of glass beads were added to each individual culture block and whole cell lysates were prepared using a vortex for 5 minutes at 4°C. Glass beads and cellular debris were pelleted by centrifugation. Lysates were removed from the 96-well culture block, transferred to 96-well Millipore filter plates (MHVB-N45) pre-loaded with 25 µl of GT-Sepharose and incubated with gentle agitation for 1 h at 4°C. Beads were washed extensively with 200 µl of lysis buffer D (without triton X-100). GST-kinases were eluted with a 10 mg/ml solution of reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8.0. 15 µl of each elution was combined with 1 µg of purified recombinant Nup53p (2.4.2) in a 96-well Costar plate (Corning) preloaded with 16 µl of 2X Kinase Buffer (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM $CaCl_2$, 2 mM ATP, and

[γ-^{32}P]-ATP). The kinase reaction was allowed to proceed for 30 min at 30°C. The reaction was stopped with the addition of 10 µl of 4X SDS-sample buffer, and boiled for 5-minutes. 20 µl from each reaction well was separated on 10% SDS-polyacrylamide gels. Gels were dried and used to expose autoradiographic films. Positive hits (Nup53p-phosphorylating kinases) were confirmed by duplicating the purification and kinase assays from the prelimary screen.

2.7 *In vitro* phosphorylation of Nup53p

1 µg of either purified recombinant GST-Hrr25p or GST-hrr25-K38A (2.4.2) was incubated with 1.5 µg of purified, recombinant Nup53p (2.4.2) in the presence of Kinase Buffer (Table 2-2) for 1 h at 30°C. Kinase reactions were stopped by the addition of 4X SDS-sample buffer followed by boiling for 5 min. Reaction products were separated by SDS-PAGE. Gels were dried and exposed to autoradiographic film to detect the incorporation of [γ-^{32}P] into Nup53p.

2.8 Fluorescence microscopy

GFP, GFP^{+}, YFP and RFP fusion proteins were visualized in live cells with either an Olympus BX-50 fluorescence microscope equipped with a SPOT digital camera or a Zeiss LSM 510 confocal microscope. Prior to viewing, yeast strains were grown under the following conditions. Strains harboring the plasmid pPS1069 (*KAP121-GFP [TRP1]*) were grown in SM lacking tryptophan to an OD_{600} of 0.3. Truncations of Nup53p in the pGFP-C-fus vectors were grown in SM lacking uracil to an OD_{600} of 0.3. Yeast strains harboring the plasmids pGFP-TUB1, pKAP121-GFP, pPHO4-NLS-GFP,

pPHO4-NLS*-GFP, pcNLS-GFP, pRPL25-NLS-GFP, pRG-NLS-GFP or pSTE12-NLS-GFP were also grown to an OD_{600} of 0.3 in SM media. If cell cycle arrests were required, the cells were transferred to YPD for 4 h before addition of arresting reagent (see 2.14). Genomically tagged strains were grown in YPD to an OD_{600} of 0.3 before viewing.

In all cases where an LSM510 confocal microscope was used, an Argon/2 laser emitting at 488 nm was employed to excite GFP, GFP^+ and YFP and the fluorescent emission was collected through a long pass filter (LP 505 nm). Alternatively, for the colocalization of Hrr25-GFP and Spc42-RFP, multitrack imaging was used. For these experiments, GFP emission was collected through a a bandpass filter (BP 505-530 nm). RFP was excited with a He 543 nm laser and the resulting image collected through a long pass 560 nm filter (LP 560). All images shown are 0.7 μm sections.

2.9 Immunofluorescence

Immunofluorescence microscopy was performed as described in Marelli et al. (2001) using affinity purified polyclonal rabbit anti-Nup53p antibodies (Marelli et al., 1998) and Cy3-conjugated, donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

2.10 Affinity purification of pA-fusions

2.10.1 For Figure 3-2

250 ml cultures of yeast strains synthesizing either Kap121-pA (KP121pA/NP170YFP) or Nup170-pA (NP170pA) were grown to an OD_{600} of 1.0. All of

the following steps were performed at 4°C. The cells were harvested by centrifugation and resuspended in 12 ml Lysis Buffer C. Cells were then lysed by passaging them three times through a French Press chamber at 1000Ω, followed by the addition of 12 ml of Lysis Buffer C containing 40% dimethyl sulfoxide and 2% triton X-100. Lysates were cleared by consecutive centrifugations at 11,300 x g for 20 min and 311,000 x g for 45 min. The supernatant was incubated overnight with 50 µl IgG-Sepharose beads (Amersham Biosciences), washed extensively with Lysis Buffer C and bound proteins were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated with trichloracetic acid (10% v/v). Proteins were solubilized in SDS-PAGE sample buffer, separated by SDS-PAGE and transferred to nitrocellulose for Western blotting.

2.10.2 For Figures 4-4 and 5-6

500 ml cultures of yeast strains synthesizing protein-A (pA) fusion proteins (NP53pA, NP53pA transformed with pAM4-1 (*HA-HRR25*), KP121pA, NP170pA, NIC96pA, and KP121pA transformed with pPHO4-NLS-GFP) were arrested as described in Section 2.14. Isolation of protein A fusions from cell lysates using IgG-Sepharose chromatography was performed as in 2.10.1. Following isolation, bead bound complexes were washed extensively with Lysis Buffer C followed by two additional washes in Lysis Buffer C containing 50 mM $MgCl_2$. Bound proteins were then eluted with a step gradient of $MgCl_2$ (200 mM, 500 mM, 1000 mM and 2000 mM) or by 0.5 M acetic acid, pH 3.4. Elutions were treated as in 2.10.1.

2.10.3 For Figure 3-2

Immunoprecipitations of Nup53-GFP and Δ405-430-GFP were performed as follows. A *nup53Δ* strain (NP53-A2) containing either pNP53-GFP or pΔ405-430-GFP was grown and lysates prepared as in 2.10.1. Supernatants were incubated overnight with anti-Nup53p polyclonal antibodies, followed by a 1 h incubation with 50 μL of Protein-G Sepharose beads (Amersham Biosciences). The beads were collected, washed, and bound proteins were eluted with a step gradient of 50, 200 and 500 mM $MgCl_2$, followed by 0.5 M acetic acid (pH 3.4). Elutions were treated as in 2.10.1.

2.11 Western blotting

Western blots were performed as follows: Post-transfer nitrocellulose membranes were blocked with 5% skim milk powder in PBS containing 0.1% tween-20. Specific rabbit polyclonal antibodies were used to detect GST, Nup53p, Kap121p (Marelli et al., 1998), Nup49p, Nup57p (Grandi et al., 1995; kindly provided by E.C. Hurt, University of Heidelberg, Germany), GFP (provided by M. Rout, The Rockefeller University; also used to detect YFP), Clb2p (Santa Cruz Biotechnology), and Gsp1p. Pds1-HA was detected using monoclonal 12CA5 antibodies. HA-Hrr25p was detected using HRP-conjugated primary antibodies, 3F10 (Roche). Protein A chimeras were detected using a rabbit IgG (ICN Pharmaceuticals, Aurora, OH). Either HRP-conjugated, donkey anti-rabbit or sheep anti-mouse secondary antibodies and the ECL system (Amersham Biosciences) were used to detect primary antibodies.

For making whole cell lysates, cells derived from 1 ml of culture were washed with H_2O and sonicated for 20-30 sec in 50 μl of SDS-PAGE sample buffer. Samples were then incubated at 75^{0}C for 15 min and proteins were separated by SDS-PAGE.

2.12 Sequence alignments

Alignments of the various NLS sequences indicated were performed online at the European Bioinformatics Institute, http://www.ebi.ac.uk/ using ClustalW.

2.13 Densitometry

The relative amounts of Nup53p released from GST-Hrr25p was quantitated by densitometry using a BioRad GS800 Densitometer and analyzed using QuantityOne software (BioRad). Densitometry measurements were acquired as OD/mm^2 units and normalized by dividing the values for release fractions by the total Nup53p bound to GST-Hrr25p. The resulting values were multiplied by 100 to calculate the percentage of Nup53p released.

2.14 Procedures for cell cycle arrest

Cells were grown in YPD to an OD_{600} of 0.3 and treated with 1) 5 μg/ml α-factor (Sigma) for 2 h to arrest in G1-phase, 2) 100 mM hydroxyurea (Sigma) for 2.5 h to arrest in S-phase, or 3) either 12.5 or 15 μg/ml nocodazole (Sigma) or 30 μg/ml benomyl (Sigma) for 2.5 h to arrest in G2/M (Hardwick and Murray, 1995). Arrests were confirmed visually by microscopy or by FACS analysis. To arrest both cdc26Δ and cdc15-2B strains in mitosis, cells were shifted from 23^{o}C to 37^{o}C for 3 h (Hartwell, 1971;

Marelli et al., 1998). For synchronous experiments, cells were grown to an OD_{600} of 0.3 and synchronized in G1 with the addition of α-factor to the growth media for 2 h. Cultures were collected and washed 3X with fresh YPD and released at the indicated temperatures with or without the addition of nocodazole.

2.15 Hrr25^{Degron} depletion and cdk1-as1 inhibition

To deplete Hrr25^{Degron}, KKY387 cultures were grown in YP-Gal and synchronized in G1. Cells were pelleted by centrifugation and resuspended in YPD with fresh α-factor (10 μg/ml) for an additional 2 h. Cells were then released into YPD (a similar procedure is described in Kafadar et al., 2003).

cdk1-as1 has a mutation in its kinase active site which allows for the selective incorporation of a bulky ATP analogue (1 NM PPI) which inhibits its kinase activity. To inhibit cdk1-as1, the yeast strain expressing the *cdk1-as1* allele (DOM0030) was arrested in G2/M and 1NM PPI was added to a final concentration of 10 μg/ml (see Bishop et al., 2002; Ubersax et al., 2003).

2.16 Budding profiles, spindle and FACS analysis

DF5 and KP121-34 strains were grown to an OD_{600} of 0.3 at 23°C. Cultures were split, half were shifted to 37°C and half retained at 23°C. Samples were taken after 3 h and fixed in 3.7% formaldehyde to determine budding profiles or prepared for FACS analysis. In a similar experiment, DF5, NP53-FL53 and NP53-Δ405-430 strains were grown to an OD_{600} of 0.4 and also prepared as above. Cells were sonicated briefly and scored as single/small budded cells (less than 50% of the diameter of the mother cell),

large budded, or multibudded. 300 cells were counted for each trial (n=3). FACS analysis was performed as previously described (Basrai et al., 1996). Data were collected on a FACS® Scan (Becton Dickinson, San Jose, CA) and analyzed using CellQuest.

Spindle length was evaluated at 23°C in DF5 and KP121-34 strains transformed with pGFP-TUB1 following release from α-factor arrest as described in Harvey and Kellogg, (2003). Percentages of short or long spindles were determined from 150-200 cells per time point.

2.17 Kap121-GFP import assay

Similar to the protocol described in Shulga et al. (1996), DF5 and NP53-Δ405-430 strains transformed with pKAP121-GFP were used to evaluate the rate of Kap121-GFP import at different points in the cell cycle. Logarithmically growing cells were arrested as described in 2.14. Cells were then collected by centrifugation, washed twice with YPD lacking glucose (YP) and resuspended in YP containing 100 mM 2-deoxyglucose (Sigma). Images were acquired at different time points after addition of 2-deoxyglucose at 23°C. To evaluate relative rate differences in Kap121-GFP import, cells were scored for nuclear accumulation of fluorescence and results were represented graphically as the percentage of cells showing a nuclear signal versus time. In 3 independent experiments, 50 cells were counted at each time point.

Chapter III: *Karyopherins in nuclear pore biogenesis: a role for Kap121p in the assembly of Nup53p into the nuclear pore complex*[*]

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3.1 Overview

In yeast, the assembly of new NPCs occurs throughout the cell cycle until they are divided between the forming daughter nuclei during telophase (Winey et al., 1997). This suggests that the machinery for the assembly of NPCs functions continuously. In the study presented here we have examined the mechanism by which one FG-nup, Nup53p, is assembled into NPCs. We show that the karyopherin Kap121p functions in the targeting and assembly of the nucleoporin Nup53p into NPCs by recognizing a nuclear localization signal in Nup53p. This karyopherin-mediated function can also be performed by the Kap95p/Kap60p complex if the Kap121p binding domain of Nup53p is replaced by a classical NLS, suggesting a more general role for karyopherins in NPC assembly. At the NPC, neighboring nucleoporins bind to two regions in Nup53p. One nucleoporin, Nup170p, associates with a region of Nup53p that overlaps with the Kap121p binding site and we show that they compete for binding to Nup53p. We propose that once targeted to the NPC, dissociation of the Kap121p/Nup53p complex is driven by the interaction of Nup53p with Nup170p. At the NPC, Nup53p exists in two separate complexes, one of which is capable of interacting with Kap121p and another that is bound to Nup170p. We propose that fluctuations between these two states drive the binding and release of Kap121p from Nup53p, thus facilitating Kap121p's movement through the NPC.

3.2 Results

3.2.1 Defining the Kap121p binding domain of Nup53p.

The specific interaction between Kap121p and the FG-nup Nup53p has led to the proposal that this nup plays a specific, perhaps regulatory, role in Kap121p-mediated import (see Chapter IV). The FGs in Nup53p are organized differently than the more common FXFG or GLFG peptide-repeat patterns found in other FG-nups. In light of these observations, we have dissected the Kap121p-Nup53p interaction in an attempt to define the region of Nup53p responsible for Kap121p binding. To do this, N- and C-terminal deletions of Nup53p were synthesized as GST fusions in *E. coli* (Fig. 3-1) and the binding of Kap121p to these fusion proteins was evaluated using a blot overlay assay previously used to detect interactions between Kap121p and a number of FG-nups including Nup53p (Marelli et al., 1998). As shown in Fig. 3-2A, Kap121-pA (containing a C-terminal protein A tag) derived from yeast cytosol bound specifically to GST-Nup53p. Kap121-pA also bound GST-fusions containing amino-acid residues 203-475 and 374-475 of Nup53p but failed to bind either of the C-terminal deletions containing residues 1-297 or 1-375. Further deletion analysis of the 374-475 region identified residues 401-448 as a minimal Kap121p interacting region. A direct interaction between this region of Nup53p and Kap121p was confirmed by an *in vitro* solution binding assay using the GST-401-448 protein and recombinant Kap121p. As shown in Fig. 3-2B, Kap121p bound specifically to GST-401-448, but not to GST alone.

Several other observations support the conclusion that residues 401-448 of Nup53p are necessary for binding Kap121p. First, a deletion mutant lacking residues

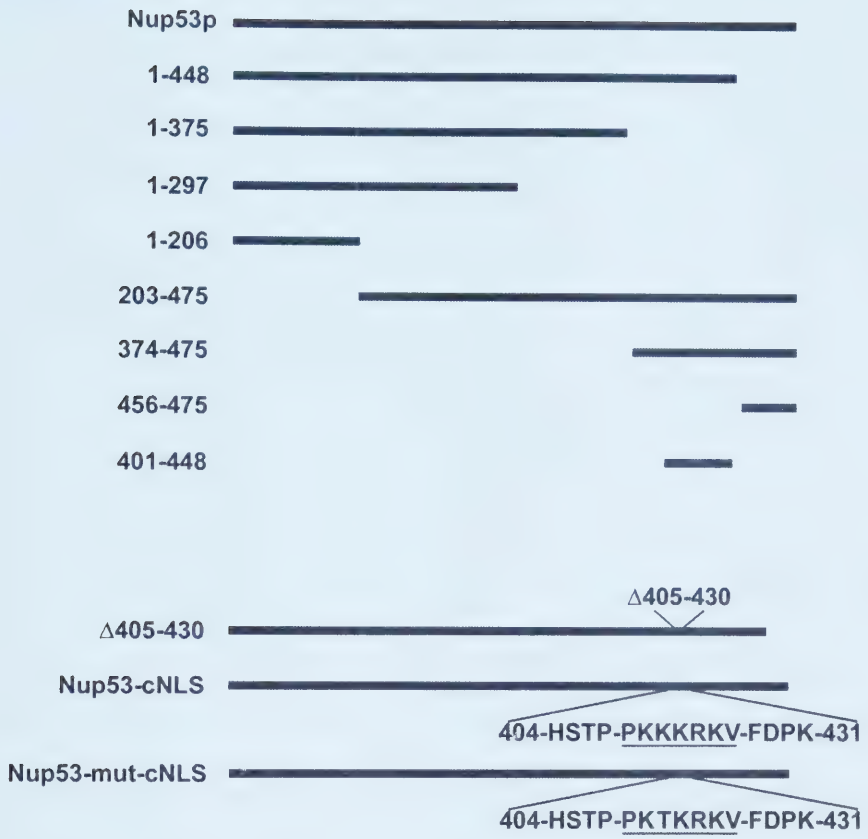

Figure 3-1. Truncations of Nup53p

N- and C-terminal truncations of Nup53p were designed as shown with amino-acid residues listed on the left. Δ405-430 indicates the deletion of these residues. The Nup53-cNLS and Nup53-mut-cNLS constructs contain insertions encoding either the SV40 T-antigen cNLS (underlined) or a mutant of the cNLS (mut-cNLS) in place of residues 405-430.

405-430 failed to bind Kap121-pA in overlay assays (Fig. 3-2A). In addition, by two separate criteria this deletion mutant failed to interact with Kap121p when expressed in yeast. First, when Nup53p or the 405-430 deletion mutant were affinity purified from yeast lysates, Kap121p was associated with wild type Nup53p, but not the 405-430 deletion mutant (Fig. 3-2C). Second, we showed that the NE association of Kap121-GFP with Nup53p was dependent on residues 405-430. To do this, we took advantage of a previous observation that removal of Nup53p from the NPC caused a visible reduction in steady-state levels of Kap121p associated with NPCs (Marelli et al., 1998, see Fig. 3-3A). This phenotype was most striking in cells lacking both Nup53p and a structurally related protein Nup59p. Introduction of Nup53p into this strain reestablished NE binding of Kap121p to approximately wild type levels (Fig. 3-3B; Marelli et al., 1998). However, as shown in Fig. 3-3C, the deletion of residues 405-430 from Nup53p reduced its ability to recruit Kap121p to the NPC. As discussed below, a significant portion of this Nup53p mutant can associate with the NPC suggesting that its inability to function as a binding site for Kap121p was not due to a complete mislocalization of the mutant protein. Together, these observations suggest that the Kap121p binding domain (KBD) of Nup53p lies between residues 401-448 in a region lacking FG-repeats.

3.2.2 Kap121p assists in the targeting of Nup53p to the NPC

Analysis of the Nup53p KBD (Fig. 3-4A) revealed that it appeared similar to known or potential NLSs in several Kap121p specific cargos including Pho4p (Kaffman et al., 1998a), Spo12p (Chaves and Blobel, 2001), Ste12p (Leslie et al., 2002) and Yap1p (Isoyama et al., 2001). We tested if the KBD of Nup53p could function in such a

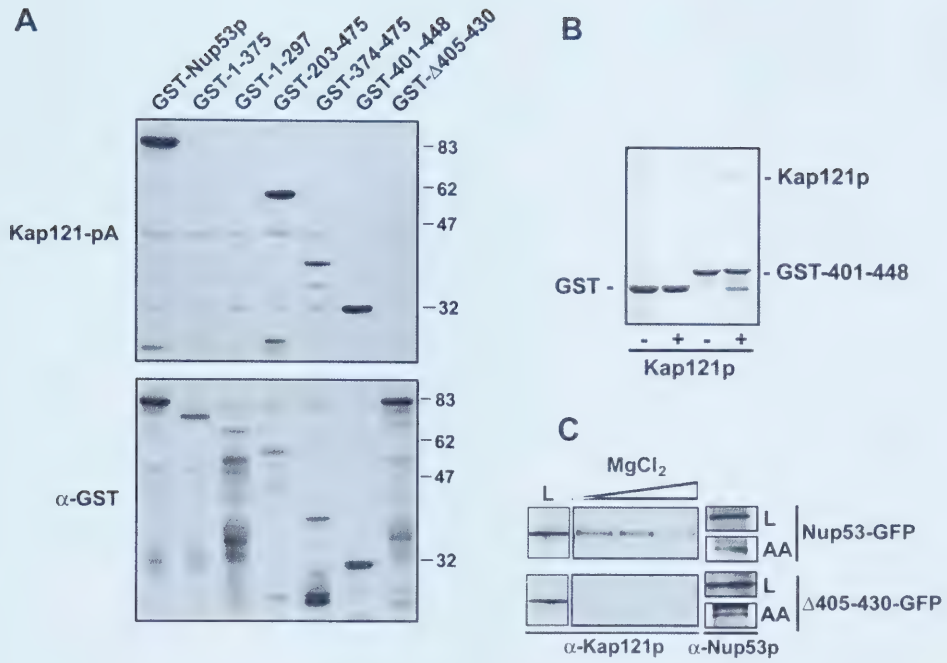

Figure 3-2. Defining the Kap121p binding domain in Nup53p.

(A) The truncations indicated were synthesized as GST fusions in *E. coli* and total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with a cytosol prepared from a yeast strain (KP121pA) producing Kap121-pA or an anti-GST antibody (α-GST) to confirm the positions of the fusion proteins. Molecular mass markers are shown in kDa. (B) GST (lanes 1 and 2) and GST-401-448 (lanes 3 and 4) were synthesized in *E. coli* and immobilized on GT-Sepharose. Beads were incubated with buffer alone (-) or purified Kap121p (+), washed, and eluted with SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and visualized with Coomassie blue. (C) Immunoprecipitations of Nup53-GFP and Δ405-430-GFP were performed from a *nup53Δ* strain transformed with either pNP53-GFP or pΔ405-430-GFP. Anti-Nup53p antibodies were incubated with yeast extracts from these strains, followed by the addition of Protein-G beads. The beads were collected, washed, and eluted with a gradient of 50, 200 and 500 mM $MgCl_2$ and lastly with 0.5 M acetic acid, pH 3.4 (AA). Proteins in the eluted fractions (50% of total) were separated by SDS-PAGE, transferred to nitrocellulose and probed with rabbit polyclonal anti-Kap121p (α-Kap121p) or anti-Nup53p (α-Nup53p) antibodies. *L* represents 0.05% of the load fraction. ("C" contributed by T. Makhnevych).

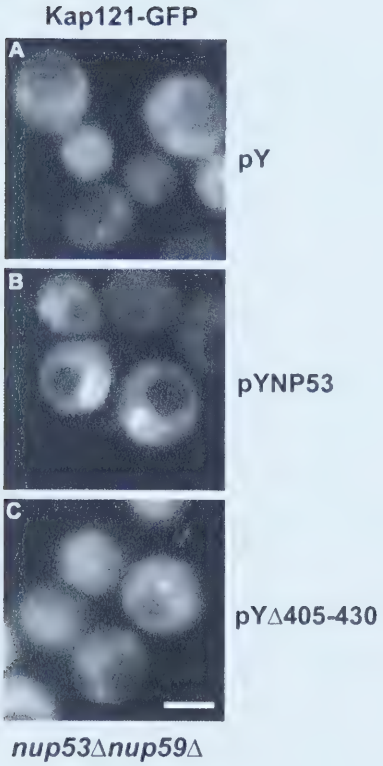

Figure 3-3. Kap121p interacts with the KBD at the NPC.

A plasmid borne copy of *KAP121-GFP* was co-introduced with either (A) an empty plasmid (pY), (B) a plasmid containing *NUP53* (pYNP53), or (C) a plasmid containing *NUP53* lacking the coding region for the KBD, *Δ405-430* (pYΔ405-430), into a haploid yeast strain lacking both *NUP53* and *NUP59* (NP53/NP59-2.1). The distribution of Kap121-GFP was determined by fluorescence microscopy. Bar, 5 μm.

capacity by constructing a reporter protein consisting of the Nup53p KBD (amino acid residues 401-448) fused to GFP (KBD-GFP). When expressed in wild type cells, the KBD-GFP concentrated in the nucleus (Fig. 3-4B). Moreover, the import of this reporter was mediated by Kap121p as cells harboring a *KAP121* temperature-sensitive (ts) allele (*kap121-41*) failed to import the KBD-GFP fusion (Fig. 3-4B).

Our observation that the Nup53p KBD could be imported by Kap121p suggested that the interactions between these proteins were not restricted to events occurring at the NPC. Moreover, we have previously shown that overproduced Nup53p will enter the nucleus by a Kap121p-dependent mechanism (Marelli et al., 2001). We hypothesized that the Kap121p-mediated import machinery may play a role in the targeting of cytoplasmic Nup53p (arising either from newly synthesized Nup53p or by release from existing NPCs) to the NPC for its assembly into these structures. Several experiments were performed to test this potential mechanism. First, we tested whether a mutation in Kap121p would affect the ability of endogenous Nup53p to associate with NPCs. For these experiments *NUP53* was genomically tagged at the 3' end of its ORF with the coding region of *GFP* in wild type and *kap121-41* ts strains. The Nup53-GFP in wild-type cells was visible in a defined punctate pattern along the nuclear periphery with no visible cytoplasmic staining (Fig. 3-4C). However, in the strain lacking fully functional Kap121p (*kap121-41*) we observed, in addition to a NE signal, a clearly visible pool of cytoplasmic Nup53-GFP (Fig. 3-4C). By comparison, a Nup59-GFP chimera was localized to the NPC in wild-type cells and this localization was not altered in the

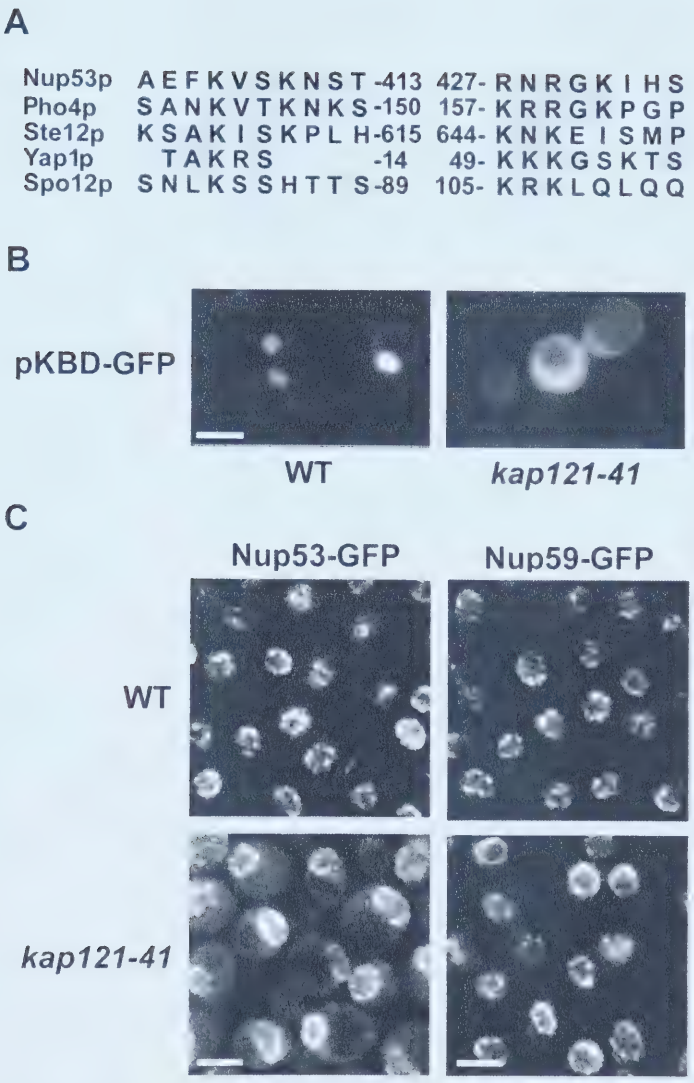
A
Nup53p A E F K V S K N S T -413 427- R N R G K I H S
Pho4p S A N K V T K N K S -150 157- K R R G K P G P
Ste12p K S A K I S K P L H -615 644- K N K E I S M P
Yap1p T A K R S -14 49- K K K G S K T S
Spo12p S N L K S S H T T S -89 105- K R K L Q L Q Q
B
pKBD-GFP
WT
kap121-41
C
Nup53-GFP
Nup59-GFP
WT
kap121-41

Figure 3-4. Kap121p functions in the efficient localization of Nup53p to the NPC.

(A) Sequence alignment of the Nup53p KBD with NLS-containing regions of Pho4p, Ste12p, Yap1p and Spo12p. Identical and similar amino-acid residues in two or more sequences are shaded. Residue numbers are shown. (B) A plasmid expressing the coding region for the KBD of *NUP53* fused to *GFP* (pKBD-GFP) was introduced into a DF5 strain (WT) and the strain KP121-41 containing a temperature-sensitive allele of *KAP121 (kap121-41)*. The distribution of KBD-GFP was monitored by fluorescence microscopy. (C) The distribution of Nup53-GFP and Nup59-GFP was examined in strains containing these fusion genes expressed behind the endogenous promoters of *NUP53* and *NUP59* in both DF5 and KP121-41 strains (NP53GFP, NP59GFP, NP53GFP/KP121-41, NP59GFP/KP121-41). 0.7 μm sections are shown. Bar, 5 μm.

kap121-41 strain (Fig. 3-4C). These results are consistent with the idea that Nup53p is inefficiently targeted to the NPC in the *kap121-41* strain.

We tested what effects removing the KBD would have on the localization of Nup53p. To do this, an ORF encoding the Δ405-430 mutant C-terminally tagged with the yellow fluorescent protein (Δ405-430-YFP) was integrated at the *NUP53* locus, replacing the wild-type ORF and placing the mutant construct under the control of the endogenous promoter. An examination of the localization of Δ405-430-YFP revealed that this protein was partially mislocalized to the cytoplasm in a pattern similar to that observed for Nup53p in the *kap121-41* mutant (Fig. 3-5B). By comparison, Nup53-YFP was visible exclusively at the nuclear rim in a punctate pattern (Fig. 3-5A). These data support the idea that the KBD of Nup53p and its recognition by Kap121p play a role in the efficient localization of Nup53p to the NPC. However, it remained to be determined whether this reflects a unique property of the Kap121p/Nup53p complex or rather the general property of a kap targeting its cargo to the NPC. To address this question, we asked whether another kap could rescue the targeting of Nup53p to the NPC. To do this, we changed the specificity of the kap-binding site in Nup53p by replacing the KBD with a nuclear localization signal (cNLS) recognized by the Kap60p/Kap95p heterodimer. Strikingly, the resulting construct was efficiently targeted to the NPC in a manner similar to wild-type Nup53p (Fig. 3-5C). Moreover, a Nup53-cNLS-GFP construct was also efficiently targeted to the NPC in the *kap121-41* ts strain (Fig. 3-5I,J). In contrast, a non functional mutant cNLS did not rescue the targeting defect (Fig. 3-5D). It was also apparent from our results that even in the absence of the KBD a portion of the Δ405-430-YFP associates with the NPC. Hence the ability of Nup53p to associate with other nups in the NPC is not

Figure 3-5. Binding to a kap assists in the assembly of Nup53p into the NPC. (A, B, C and D) Strains were constructed where the ORF of one of the two *NUP53* genes in a diploid DF5 strain was replaced with either an ORF encoding *NUP53-YFP* (NP53YFP), *Δ405-430-YFP* (Δ405-430-YFP), *NUP53-cNLS-YFP* (NP53-cNLS-YFP), and *NUP53-mut-cNLS-YFP* (NP53-mNLS-YFP). The distribution of the resulting fusion proteins was monitored by fluorescent confocal microscopy. (E, F, G and H) The effects of additional copies of wild-type *NUP53* on the distribution of Nup53-YFP, Δ405-430-YFP, Nup53-cNLS-YFP and Nup53-mut-cNLS-YFP were examined following introduction of the plasmid pRNP53 (*CEN/URA3/NUP53*). (I and J) *NUP53-GFP* and *NUP53-cNLS-GFP* were expressed from pNP53-GFP and pNP53-cNLS-GFP in the strain KP121-41, and the GFP fusions visualized as above. Bar, 5 μm.

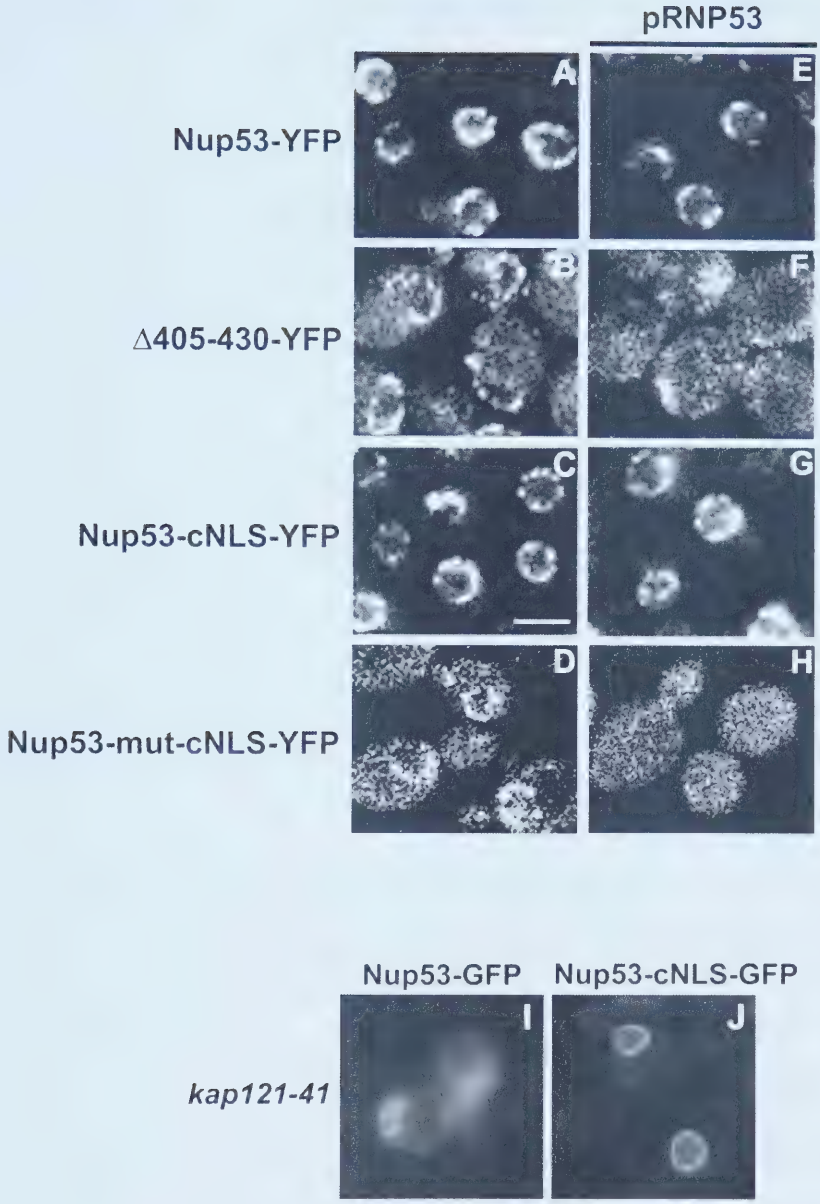

Figure 3-5. Binding to a kap assists in the assembly of Nup53p into the NPC.

strictly dependent on its interactions with Kap121p. We would predict that if its interaction with Kap121p facilitates Nup53p's association with other nups, the presence of a kap-binding domain should provide wild-type Nup53p with a competitive advantage over constructs lacking the KBD for assembly into the NPC. To determine whether this is the case, surplus copies of *NUP53* were introduced by transformation with the plasmid pRNP53 into strains expressing *NUP53-YFP*, *Δ405-430-YFP*, *NUP53-cNLS-YFP*, and *NUP53-mut-cNLS-YFP*. As shown in Fig.5E and 5G, the presence of wild-type Nup53p did not appreciably affect the association of Nup53-YFP or Nup53-cNLS-YFP with the NPC, but it blocked the binding of the Δ405-430-YFP and the Nup53-mut-cNLS-YFP constructs to the NPC (Fig. 3-5F and 5H). These results demonstrated that the association of Nup53p with a kap played a distinct and advantageous role in the localization of Nup53p to the NPC.

3.2.3 The *in vivo* distribution of truncations of Nup53p

Once present within the context of the NPC, the steady-state association of Nup53p with this structure is determined by its association with nups. To define domains within Nup53p that mediate its association with other nups, we tested the ability of a series of Nup53p deletion mutants to associate with the NPC (Fig. 3-6). Each of the truncations and wild-type *NUP53* were fused to the N-terminus of *GFP* in the plasmid pGFP-C-fus. Importantly, the localization of the chimeras was examined in strains lacking endogenous Nup53p (*nup53Δ*) to eliminate competition for binding sites at the NPC. In addition, these constructs were also examined in strains lacking the nucleoporin Nup120p. In the *nup120Δ* strain, NPCs cluster to focal points along the NE allowing

NPC association to be distinguished from general NE localization (Aitchison et al., 1995). As shown in Fig. 3-6, Nup53-GFP was localized along the periphery of the nucleus in a punctate pattern in the *nup53Δ* strain and clustered to distinct foci in the *nup53Δ nup120Δ* strain (inset). A series of C-terminal deletions was examined beginning with the removal of the last 27 amino-acid residues of Nup53p. This 1-448-GFP construct, containing the KBD, exhibited an NPC associated pattern similar to that of wild-type Nup53p (Fig. 3-6). A 1- 375-GFP lacking the KBD also bound NPCs albeit exhibiting a weaker NE signal and higher levels of cytoplasmic fluorescence. In contrast, the 1-206-GFP protein did not visibly associate with the nuclear periphery and was distributed throughout the cytoplasm. A construct containing residues 202-375 was also examined and did not associate with NPCs suggesting that this region was necessary, but not sufficient, for binding the NPC.

Several N-terminal deletions were also examined (Fig. 3-6). Most strikingly, both the 203-475-GFP and 374-475-GFP proteins containing the KBD showed the same localization patterns described above for wild-type Nup53p. However, when we further deleted the KBD, the resulting protein, 456-475-GFP, was observed in a diffuse staining pattern throughout the cytoplasm. In addition, 456-475-GFP also associated with the NE and plasma membrane suggesting it was capable of binding multiple membrane systems in the cell. However, the NE membrane association does not appear to involve a stable interaction with the NPC as the protein failed to cluster with NPCs in *nup53Δnup120Δ* cells. Taken together, the deletion analysis suggests that two regions within Nup53p play a predominant role in its association with the NPC, one region lying between amino-acid residues 1-375 and a second region, containing the KBD, between residues 374-475.

Figure 3-6. The *in vivo* localization of truncations of Nup53p.
Plasmid borne copies of chimeric genes encoding full length Nup53p and the indicated truncations fused to GFP were introduced into a haploid *nup53Δ* strain (NP53-A2) and a haploid *nup53Δnup120Δ* strain where NPCs cluster (NP53/NP120-25-3). The cellular distribution of these chimeras was determined after growth to early-log phase at 23°C. Fluorescent and a combination of DIC and fluorescent confocal microscope images are shown as 0.7 μm sections for all constructs in the *nup53Δ* strain. The insets in the panels in the left column show the localization of the truncations in the *nup53Δnup120Δ* strain imaged using an Olympus BX-50 fluorescent microscope with a SPOT digital camera. Bar, 5 μm.

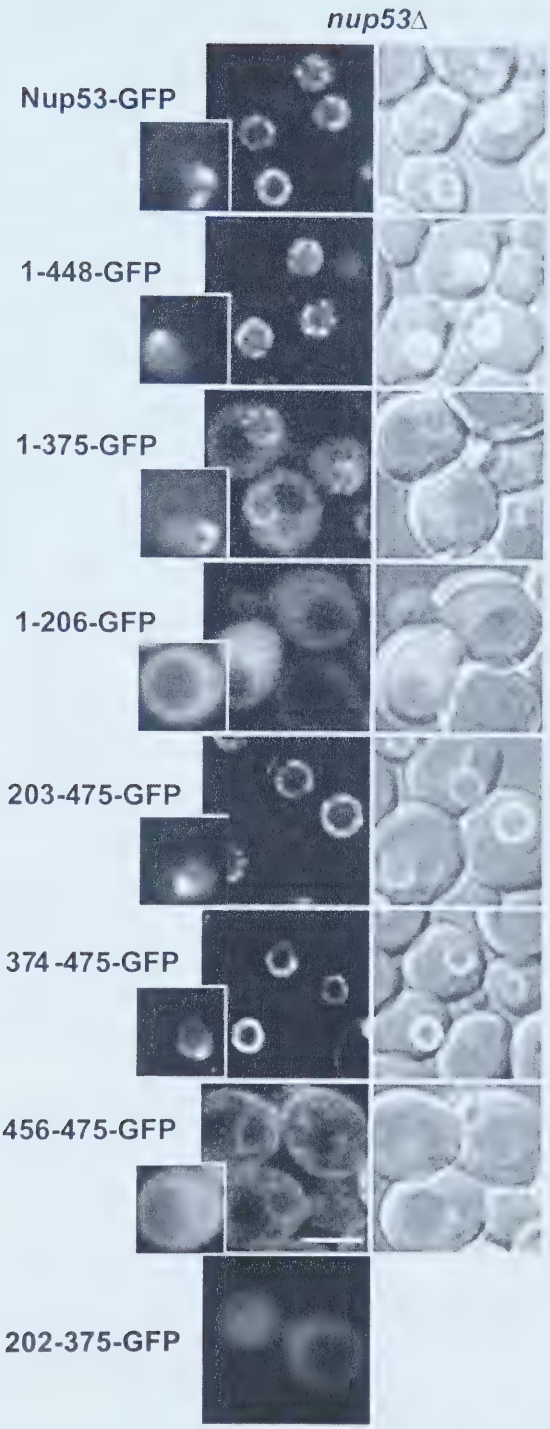

Figure 3-6. The *in vivo* localization of truncations of Nup53p.

3.2.4 Mapping interactions between Nup53p and other nups

To investigate which nups interact with the two NPC binding regions of Nup53p, we examined the localization of both 1-375-GFP and 374-475-GFP in different nup mutants. A potential candidate was Nic96p since several pieces of data supported the idea that these two nups interact. Work from Fahrenkrog et al. (2000) showed that Nic96p is bound to Nup53p affinity-purified from yeast extracts (also see Fig. 4-4). Moreover, an interaction between these proteins was detected by two-hybrid analysis (Uetz et al., 2000; Fahrenkrog et al., 2000; S. Tcheperegine and R.W. Wozniak unpublished data) and by direct *in vitro* binding experiments (Appendix, Fig. 8-1). On the basis of these observations, we examined the localization of Nup53-GFP, 1-375-GFP and 374-475-GFP in a strain harboring a ts allele of *NIC96*, *nic96-1* (Grandi et al., 1995), and lacking *NUP53* (*nup53Δ nic96-1*). In this strain, the NPC association of 1-375-GFP was clearly reduced as compared to the *nup53Δ* strain (compare Fig. 3-7A, panel B to Fig. 3-6). In contrast, both Nup53-GFP and 374-475-GFP exhibited a strong nuclear peripheral signal indicating that the distribution of these proteins were not affected by the *nic96-1* allele. These results suggest that Nic96p, either directly or indirectly, mediates the association of the 1-375 region of Nup53p with the NPC.

We have postulated that Nup170p is also an important binding partner for Nup53p at the NPC. This was based on several observations including the association of Nup170p with an isolated, Nup53p-containing subcomplex (Marelli et al., 1998) and the requirement for Nup170p in the targeting of overproduced Nup53p to the NPC (Marelli et al., 2001). Moreover, we have also observed that endogenous Nup53p is partially mislocalized in a *nup170* null mutant (Fig. 3-7C) in a pattern reminiscent of that observed

Figure 3-7. Nic96p is required to properly localize the N-terminus of Nup53p to the NPC.

(A) The subcellular distributions of Nup53-GFP and two truncations of Nup53p, 1-375-GFP and 374-475-GFP, were examined in a strain containing a temperature-sensitive allele of *NIC96*, *nic96-1*, and lacking endogenous *NUP53* (NP53/*nic96-1*) or in (B) strains lacking endogenous *NUP53* (NP53-A2) or *NUP53* and *NUP59* (NP53/NP59-2.1). Shown are fluorescent or a combination of fluorescent and DIC images obtained from 0.7 μm confocal sections of cells grown to mid-log phase at 23°C. Compare the distribution of 1-375-GFP to that of 1-375-GFP in the *nup53Δ* strain (Fig. 3-6). (C) Immunofluorescence microscopy using affinity-purified α-Nup53p antibodies was performed on W303 (WT) and *nup170Δ* (NP170-11.1) strains. Antibody binding was detected using Cy3-conjugated anti-rabbit antibodies. Bar, 5 μm.

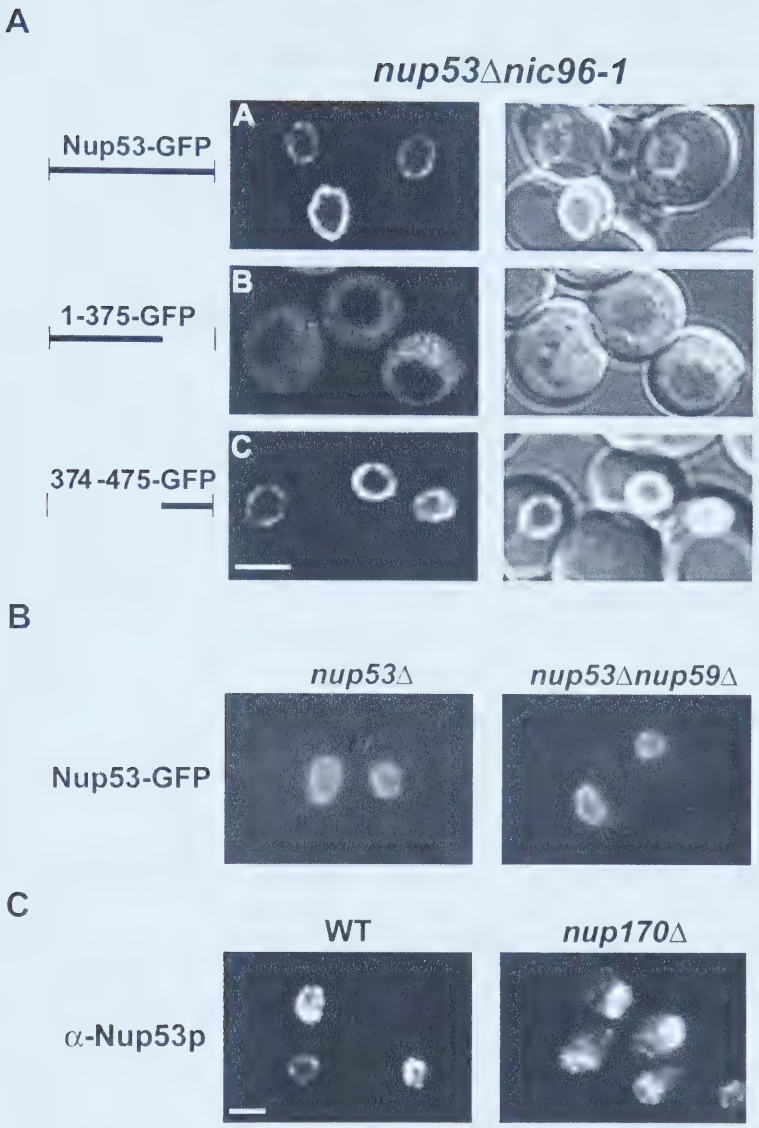

Figure 3-7. Nic96p is required to properly localize the N-terminus of Nup53p to the NPC.

in the *kap121-41* ts mutant (Fig. 3-4C). These results suggest that Nup170p plays a role in the deposition of Nup53p at the NPC. To further investigate the interactions between these nups, we mapped the region of Nup53p that binds to Nup170p using overlay assays. As shown in Fig. 3-8A, Nup170-pA derived from yeast cell lysates bound to a C-terminal region of Nup53p (amino-acid residues 374-475) containing the KBD, but did not bind to residues 1-375 and showed attenuated reactivity with the Δ405-430 construct. Consistent with these data, when the 374-475-GFP fusion protein was produced in cells lacking Nup170p (*nup170Δ*), it no longer associated with the NPC but instead was mislocalized to the cytoplasm (Fig. 3-8B). Together, these results suggest that Nup170p, either directly or indirectly, mediates the association of residues 374-475 of Nup53p with the NPC.

These results led us to investigate the possibility that Nup170p and Kap121p competed for the same binding site on Nup53p, and that this could provide a mechanism for releasing Nup53p from Kap121p at the NPC. To test this idea, overlay assays examining the binding of Nup170-pA to Nup53p were performed in the presence of recombinant Kap121p. As shown in Fig. 3-8C, increasing concentrations of Kap121p beginning at 50 nM competed for the binding of Nup170-pA to Nup53p.

We concluded from these data that *in vivo* Nup53p might exist in two separate complexes, bound either to Nup170p or to Kap121p. In light of these results, our previous data showing that Nup170p and Kap121p co-purified with Nup53-pA (Marelli et al., 1998) could represent a combination of separate Nup53p/Kap121p and Nup53p/Nup170p complexes. To test this, Nup170-pA and Kap121-pA were separately affinity-purified from yeast cells and eluted fractions were examined by Western blotting

Figure 3-8. Nup170p and Kap121p interact with an overlapping region of Nup53p. (A) Nitrocellulose membranes containing SDS-PAGE separated *E. coli* total cell lysates containing GST fusions of Nup53p and the indicated mutants, were probed with either a cell lysate derived from a yeast strain expressing *NUP170-pA* (NP170pA) or an anti-GST antibody (α-GST). The positions of molecular mass markers of the indicated size in kDa are shown on the left. (B) The subcellular distribution of the Nup170p binding domain of Nup53p was examined in WT (W303) and *nup170Δ* (NP170-11.1) strains using a 374-475-GFP fusion protein. Shown are confocal microscopy images acquired in both the fluorescent channel and a combination of the fluorescent and DIC images. Bar, 5 μm. (C) Equivalent amounts of GST-Nup53p in *E. coli* cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed separately with an equal amount of a yeast total cell lysate containing Nup170-pA and supplemented with 0, 50, or 500 nM concentrations of recombinant Kap121p. The Nup170-pA fusion was detected using HRP-conjugated IgG antibodies and ECL. (D) Total cell lysates derived from yeast strains synthesizing Nup170-pA (NP170pA) or Kap121-pA (KP121pA/NP170YFP) were immunopurified on IgG Sepharose columns. 0.05% of the unbound fraction (UB), and 50% of the protein eluted from the beads with 1 M $MgCl_2$ (B) were analyzed by immunoblotting with α-Nup53p, α-GFP, or α-Kap121p antibodies to detect the indicated protein. ("D" contributed by T. Makhnevych).

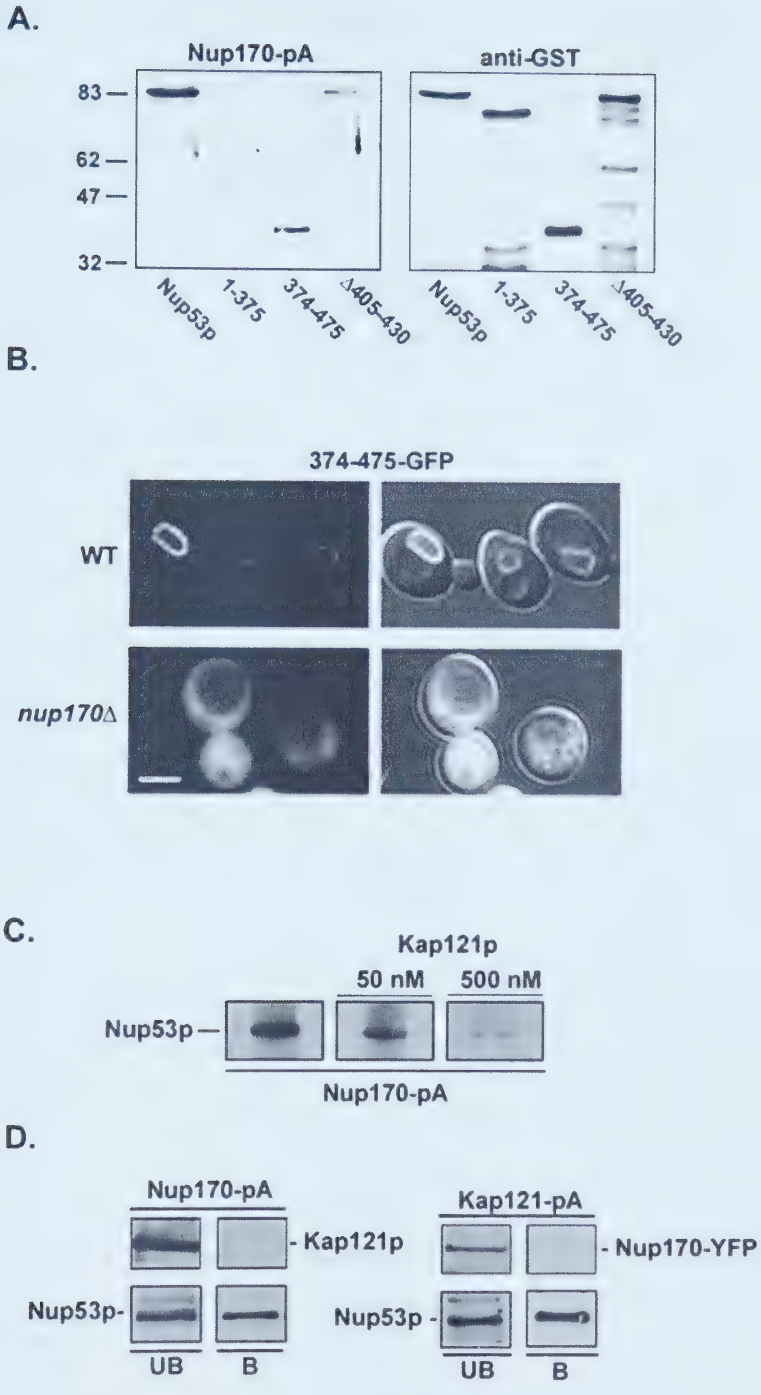

Figure 3-8. Nup170p and Kap121p interact with an overlapping region of Nup53p.

(Fig. 3-8D). Nup53p was detected in fractions eluted from both the Nup170-pA and Kap121-pA. In both cases, Nup53p was also detected in the unbound fraction. However, Nup170p was not bound to Kap121-pA nor was Kap121p detected in fractions eluted from Nup170-pA.

3.3 Discussion

Our studies examining the specific interaction of Nup53p with Kap121p have uncovered a role for karyopherins in the targeting and assembly of nucleoporins into NPCs. We propose a model in which Kap121p, in addition to binding Nup53p at the NPC, can bind to a soluble, cytoplasmic pool of Nup53p, potentially derived from either newly synthesized Nup53p or from that released from existing NPCs, and target it to NPCs where it associates with other nups. The binding of Kap121p to Nup53p occurs through a region of the nup that lacks FG repeats (amino acid residues 401-448), but can function autonomously as an NLS (Fig. 3-4B). It remains to be determined, however, whether Nup53p shares the same binding site on Kap121p as the prototypical cargo NLSs or if it binds to a unique site.

We propose that once bound to free Nup53p, Kap121p assists in its assembly into NPCs (see model in Fig. 3-9, step A). While Kap121p is not strictly necessary for Nup53p to bind NPCs, it is clear that the efficient targeting of Nup53p to the NPC requires both functional Kap121p and the KBD of Nup53p. Moreover, the KBD imparts a distinct competitive advantage on Nup53p for integration into the NPC over mutant constructs lacking only the KBD (Fig. 3-5). One interpretation of these results is that Kap121p can act as a chaperone that maintains Nup53p in a conformation capable of binding more efficiently to its neighboring nups. Consistent with this idea, the competitive advantage provided by the KBD is not solely a property of this peptide but rather appears to be related to its ability to bind a karyopherin. We show that the KBD can be replaced with a cNLS recognized by the Kap95p/Kap60p complex and the resulting construct is assembled into NPCs as efficiently as wild-type Nup53p (Fig. 3-5).

On the basis of these results it seems likely that other kaps may perform similar assembly functions for other nups.

One can envision that nups containing NLSs, and possibly other kap binding sites such as FG repeats, could be recognized by kaps following their synthesis and that these interactions play a role in their assembly into NPCs. This hypothesis is, however, inherently difficult to test since, unlike Nup53p, most of the kap binding nups bind multiple kaps and thus individual kap mutants are unlikely to cause assembly defects. This is the case for mammalian Nup153, which contains both FG-repeats and an M9 NLS-like region (Nakielny et al., 1999). Nup153 appears to represent a class of mobile nups (Daigle et al., 2001; Rabut et al., 2004; Griffis et al., 2004; see section 1.3.1.4), and it has been suggested that Kap-β2/transportin 1, by binding the M9 NLS-like region, plays a role in mediating its movement to the NPC (Nakielny et al., 1999). However, this potential function for Kap-β2/transportin 1 remains to be established. Other nups including mammalian Nup98 (Fontoura et al., 2000), Tpr (Bangs et al., 1998) and yeast Nic96p (Grandi et al., 1995) also contain functional NLSs and it will be of interest to determine their roles in NPC assembly and dynamics.

At the NPC, two domains of Nup53p are required for its steady state association with the NPC. Deletion analysis mapped these binding sites within amino-acid residues 1-375 and a region encompassing the KBD between residues 374-475. Residues 1-375 appear to interact either directly or indirectly with Nic96p. We observed that the 1-375 deletion construct failed to bind the NPC and was mislocalized to the cytoplasm in a strain containing a ts mutation in *NIC96* (Fig. 3-7A). This is in contrast to the 374-475 segment of Nup53p whose localization to the NPC was not affected in the *nic96-1* strain.

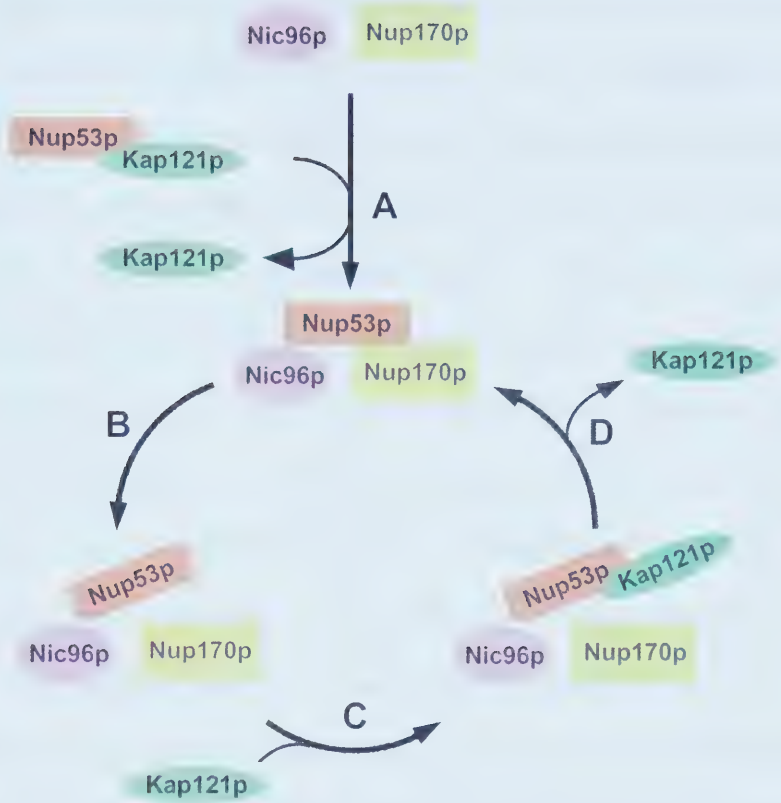

Figure 3-9. A model for the interactions of Nup53p with Kap121p at the NPC.

(A) Kap121p interacts with a soluble, cytoplasmic pool of Nup53p and assists in its direct or indirect association with Nup170p and Nic96p at the NPC. This is accompanied by the release of Kap121p from Nup53p. (B) Nup53p's association with Nup170p is thought to be transient and it can dissociate from Nup170p. (C) Dissociation from Nup170p exposes the KBD of Nup53p, allowing it to act as a binding site for Kap121p. (D) Kap121p bound to Nup53p at the NPC could then be released by stochastic changes that allow Nup53p to bind Nup170p.

The NPC localization of the 374-475 fragment was, however, strictly dependent on Nup170p (Fig. 3-8B). This observation is consistent with *in vitro* mapping data showing that Nup170p binds to this region of Nup53p (Fig. 3-8A).However, due to the nature of the overlay assays used to define these interactions, we cannot exclude the possibility that the association of Nup170p with this region of Nup53p is bridged by another nup. This could theoretically include a number of nups that interact with Nup53p (See Appendix), including Nup59p. It appears unlikely that Nup59p could mediate this interaction, however, as *nup59Δ* strains do not effect the localization of Nup53p (Fig. 3-7).

Whether direct or indirect, the association of Nup170p with Nup53p occurs through a region that overlaps with the KBD. This overlap likely reveals an important step in the incorporation of Nup53p into the NPC. We propose that once the Nup53p/Kap121p complex enters the confines of the NPC, Nup53p's association with Nup170p drives the release of Nup53p from Kap121p (Fig. 3-9, step A). Consistent with this idea, Nup170p and Kap121p compete with one another for binding to Nup53p (Fig. 3-8C). Moreover, the binding of Kap121p or Nup170p to Nup53p appears to be mutually exclusive. When purified from yeast extracts, Nup53p is bound to either Kap121p or Nup170p but not simultaneously to both (Fig. 3-8D). We envision two possible scenarios that could explain the release of Nup53p from Kap121p and its association with Nup170p. Firstly, the binding of the Nup53p/Kap121p complex to the NPC through other FG-containing nups could stimulate its dissociation, perhaps by a mechanism analogous to the accelerated release of import cargo from kaps observed upon binding to Nup1p, Nup2p, and Nup98 (Rexach and Blobel, 1995; Fontoura et al., 2000; Solsbacher

et al., 2000; Gilchrist et al., 2002; Matsuura et al., 2003). Once released, Nup53p could then bind to multiple Nup170p sites within the NPC. Alternatively, the direct binding of Nup170p to Nup53p could alter its conformation and reduce its affinity for Kap121p, leading to the release of Kap121p. By either mechanism, once bound to Nup170p, Nup53p is predicted to exhibit a lower affinity for Kap121p.

What must be reconciled with this model is that NPC associated Nup53p, through its KBD, acts as a binding site for Kap121p (Fig. 3-3) and thus some portion of NPC-bound Nup53p is predicted to be free from Nup170p. A clue to solving this conundrum may come from the results of previous work showing that Nup53p's association with Nup170p may be transient (Marelli et al., 2001). This is suggested by the observation that when overproduced, Nup53p is targeted to the NPC from where it is released and then associates with the inner nuclear membrane. This process is dependent on Nup170p, but Nup170p does not accompany the excess Nup53p to the inner nuclear membrane. Our interpretation of these results is that Nup53p can readily dissociate from Nup170p (Fig. 3-9, step B). However, what would drive their dissociation is unclear. If the off rate is sufficient, normal stochastic changes in the NPC may allow Nup53p to fluctuate between two conformational states within the NPC. In one conformation Nup53p would be free from Nup170p and capable of binding Kap121p. In the other, Nup53p would be bound to Nup170p and either unable to bind to, or exhibit a reduced affinity for, Kap121p. This again is consistent with our observation that Nup53p can be isolated in separate complexes with Kap121p or Nup170p (Fig. 3-8D). In either state, we propose that Nup53p is also tethered to the NPC, likely through a mechanism requiring Nic96p. This interaction is unlikely to interfere with Nup53p's interaction with either Nup170p or

Kap121p binding (Fig. 3-9, step C). Consistent with this idea, it is interesting to note that the same *NIC96* mutant allele (*nic96-1*) that fails to interact with Nup53p also abolishes the nuclear rim localization of Kap121p (Damelin and Silver, 2000).

In order to assume different conformations while positioned within the NPC, a necessary property of Nup53p would likely be a flexible tertiary structure. This concept is supported by recent observations by Denning and co-workers (2002) showing that Nup2p, and in particular its FG-repeat region, exists in a native unfolded structure. These data also lead the authors to propose, in another study, that this may be a general property of the FG-repeat nups (Denning et al., 2003).

The ability of Nup53p to exist in separate conformations has important implications for its role in Kap121p-mediated transport. A prediction of this model is that the release of Kap121p from its Nup53p binding site in the NPC can be facilitated by the transition of Nup53p to a Nup170p-bound state, allowing Kap121p to proceed to other nups or exit the NPC (Fig. 3-9, step D). This mechanism could occur rapidly and independently of RanGTP. However, it is also possible that RanGTP could facilitate the release of Kap121p. RanGTP can dissociate the Kap121p/Nup53p complex in vitro (Marelli et al., 1998), an effect that RanGTP has on a number of nup/import karyopherin complexes (see Allen et al., 2001 for an extensive list). This includes nups that are positioned asymmetrically or symmetrically (such as Nup53p) on the cytoplasmic and nucleoplasmic faces of the NPC. However, it is unclear whether RanGTP performs this function throughout the NPC or whether it is restricted to the nucleoplasmic face.

A mechanism by which dynamic interactions between nups can modulate their affinity for kaps may also extend to other kap-binding nups including the FG-nups. In

principle, once bound to a receptor nup, the kap could then be released by the interaction of the receptor nup with specific neighboring nups. This could be accomplished by the neighboring nups inducing a conformational change in the receptor nup that promotes a change in its affinity for the kap, or by direct competition for the kap binding site. This idea may also partially explain the apparent discrepancy between the higher affinity interactions observed between nups and kaps *in vitro* (Allen et al., 2001; Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003) and the apparent requirement for low affinity interactions necessary to accommodate the rapid flux of kaps through the NPC (Ribbeck and Gorlich, 2001). Taken a step further, one can envision that similar types of binding sites (e.g. FG repeats) could exhibit different release rates depending on their position in the NPC and their neighboring nups.

Chapter IV: *Cell cycle regulated transport controlled by alterations in the nuclear pore complex*[*]

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4.1 Overview

Our results presented in Chapter III suggest that Nup53p exists in two separate complexes at the NPC. One, which is bound to Nup170p, and the other, bound to Kap121p. It remained to be determined, however, whether these interactions reflected a dynamic between two different states, or whether they suggested that Nup53p was a part of two stable complexes. In the this Chapter, we provide support for the idea that Nup53p's interactions at the NPC fluctuate between a Kap121p-bound and Nup170p-bound state, alongside progression through the cell cycle. We propose that these molecular rearrangments provide a mechanism to explain the observation that Kap121p-mediated import is inhibited during mitosis. Specifically, our data are consistent with a model whereby during interphase Kap121p is unable to interact with Nup53p since the majority of Nup53p is bound to Nup170p. Upon entry into M-phase, the interaction between Nup53p and Nup170p is broken, exposing the KBD. We provide evidence that supports that the binding of Kap121p to the KBD inhibits the translocation of Kap121p through the NPC, and potentially causes the premature release of its cargo. The significance of this mechanism is underscored by data showing that the regulation of Kap121p–mediated import plays a key role in progression through mitosis.

4.2 Results

4.2.1 Progression through mitosis is delayed in *kap121* mutants

Kap121p is an essential yeast karyopherin for which a limited number of imported cargo molecules have been identified. Inspection of the morphology of two temperature sensitive (ts) alleles, *kap121-34* and *kap121-41* (Marelli et al., 2001; Leslie et al., 2002), revealed that cells grown at the permissive temperature contained a higher percentage of large-budded and multibudded cells as compared to their wild-type (WT) counterparts (A. Anderson and R.W. Wozniak, unpublished) suggesting a higher percentage of cells in mitosis in the mutant cell population. Consistent with this idea, FACS analysis showed that the majority of mutant cells in cultures contained a 2C or greater complement of DNA (Fig. 4-1A). Furthermore, shifting cultures to the nonpermissive temperature (37°C) for 3 h led to an increase in the number of mutant cells containing greater than 2C DNA content at 37°C (Fig. 4-1A).

To further investigate the mitotic delay exhibited by the *kap121-34* mutant, we monitored levels of the mitotic cyclin Clb2p. Upon release from α-factor induced G1 arrest, levels of Clb2p rose at similar times in both the WT and *kap121-34* cells suggesting they progressed into mitosis at similar rates. However, the length of mitosis was extended in the *kap121-34* mutant as the levels of Clb2p remained elevated in this strain for a significantly longer period of time than in WT cells (Fig. 4-1B). This mitotic delay appears to be initiated at a point near the metaphase-anaphase transition. We observe that Pds1p degradation, an APC substrate eliminated at this point, was not affected in the *kap121-34* mutant suggesting that cells normally approach this transition. However, an examination of spindle length following G1 release showed that *kap121-34*

Figure 4-1. *KAP121* mutant strains exhibit a delay in progression through mitosis. (A) FACS analysis was performed on wild-type (WT) DF5 cells and a strain harboring the *kap121-34* ts allele (KP121-34) grown at 23°C or 37°C for 3 h. The positions of 1C and 2C peaks are indicated. (B) Logarithmically growing cultures of DF5 (WT) and KP121-34 cells expressing a plasmid-borne copy of *PDS1-HA* were synchronized in G1 with α-factor (time = 0 min). α-factor was then removed and cells were grown at 23°C in YPD. Samples were taken at the indicated times and total cell lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting using antibodies directed against Clb2p (α-Clb2p), the HA tag, and a load control, Gsp1p (α-Gsp1p). (C) Cultures of DF5 (WT) and the KP121-34 cells producing tubulin-GFP were arrested in G1, released, and scored for short (1-4 μm) or long (>4 μm) spindles at the indicated times. Contributed by A. Anderson and T. Makhnevych.

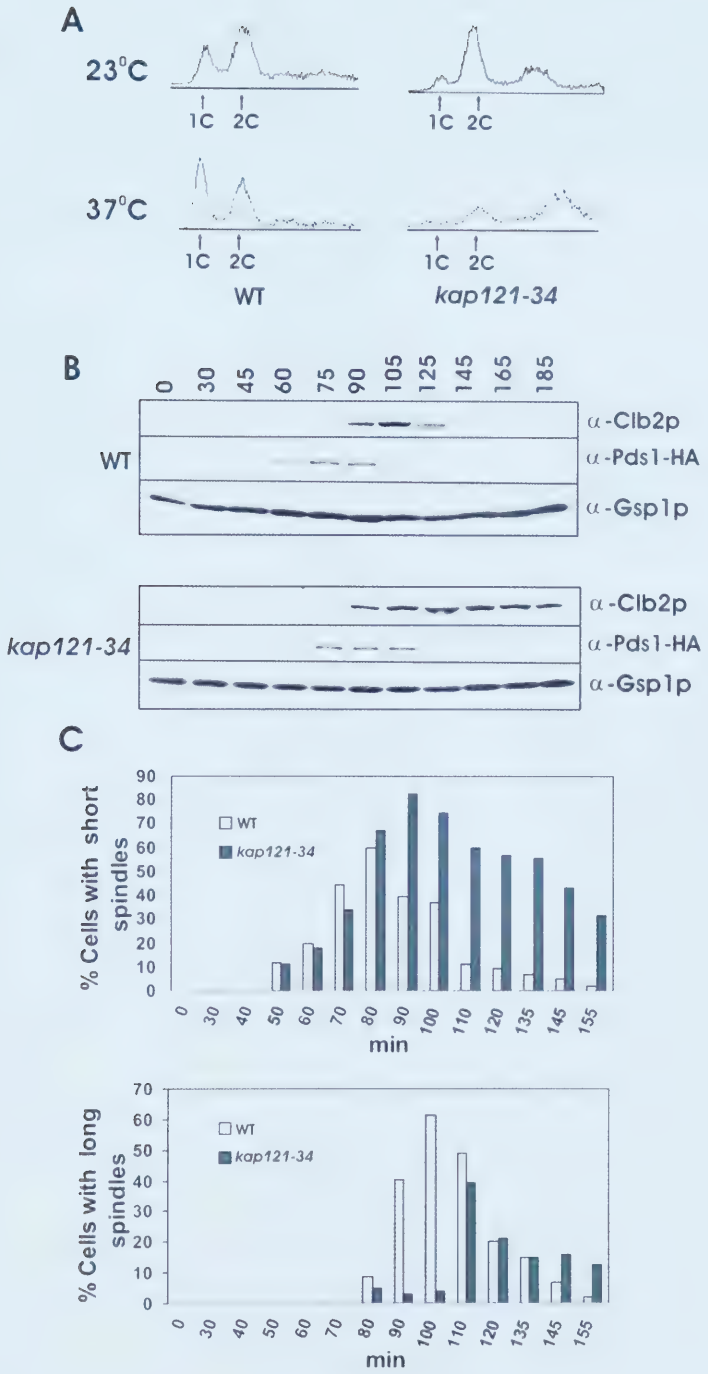

Figure 4-1 *KAP121* mutant strains exhibit a delay in progression through mitosis.

cells were delayed in their transition from short spindles (1-4 μm) to long spindles (> 4 μm; Fig. 4-1C), consistent with a mitotic delay beginning with progression from metaphase to anaphase.

4.2.2 M-phase specific regulation of Kap121p-mediated transport

On the basis of the cell cycle defects observed in the *kap121* mutants, we hypothesized that Kap121p plays a role in mediating the flux of mitotic regulators into the nucleoplasm. We envisioned two scenarios by which this could occur. In one case, nuclear levels of Kap121p cargo molecules could be regulated by either their levels of synthesis or their ability to bind Kap121p. Alternatively, the import of Kap121p specific cargos could be controlled by altering the rate of Kap121p entry, and thus its cargo, into the nucleus. In this latter case, global changes in Kap121p-mediated import are predicted to accompany progression through mitosis. To address these two possibilities, we examined the levels of Kap121p–mediated import at various stages of the cell cycle using a GFP-tagged reporter cargo. Cells containing the reporter were synchronized in G1-phase using α-factor, in S-phase using hydroxyurea, or in G2/M-phase using nocodazole, and the steady-state distribution of the reporter was examined by fluorescence microscopy. The import of two separate Kap121p-specific import reporters was examined, a Pho4-NLS-GFP protein containing the NLS of the Pho4p transcription factor (Kaffman et al., 1998a) and a Ste12-NLS-GFP fusion containing the C-terminus of the Ste12p transcription factor (Leslie et al., 2002). Both reporters were concentrated in the nucleus of cells arrested in S-phase (Fig. 4-2A). In contrast, these reporters failed to

Figure 4-2. Kap121p-mediated import is inhibited during mitosis.
(A,B, and C) DF5 cells transformed with the plasmid pPHO4-NLS-GFP, pSTE12-NLS-GFP, pcNLS-GFP, pRG-NLS-GFP, pRPL25-NLS-GFP, or pPHO4-NLS*-GFP were grown to early log phase and arrested in G1 with α-factor, S with hydroxyurea or G2/M with nocodazole as indicated. Strains harboring ts alleles of *CDC15* (cdc15-2B) or *KAP121* (KP121-34) and containing pPHO4-NLS-GFP or pPHO4-NLS*-GFP, respectively, were grown at 23°C and then shifted to 37°C for 3 h. Note, only KP121-34 cells grown at 37°C are shown in panel C. The subcellular distribution of the GFP reporters was examined using fluorescence confocal microscopy. Bars, 5 μm. (D) A yeast strain producing a protein-A tagged Kap121p chimera (KP121pA) and PHO4-NLS-GFP were arrested in S- or G2/M-phase. Kap121-pA was affinity purified from post-ribosomal supernatants using IgG-Sepharose beads. 0.05% of the proteins in the post-ribosomal supernatant (Load) and the 10% of the eluate of the IgG Sepharose beads were separated by SDS-PAGE and Western blotted using α-GFP antibodies to detect Pho4-NLS-GFP. The HRP-conjugated secondary antibodies also bind the protein-A moiety in Kap121-pA.

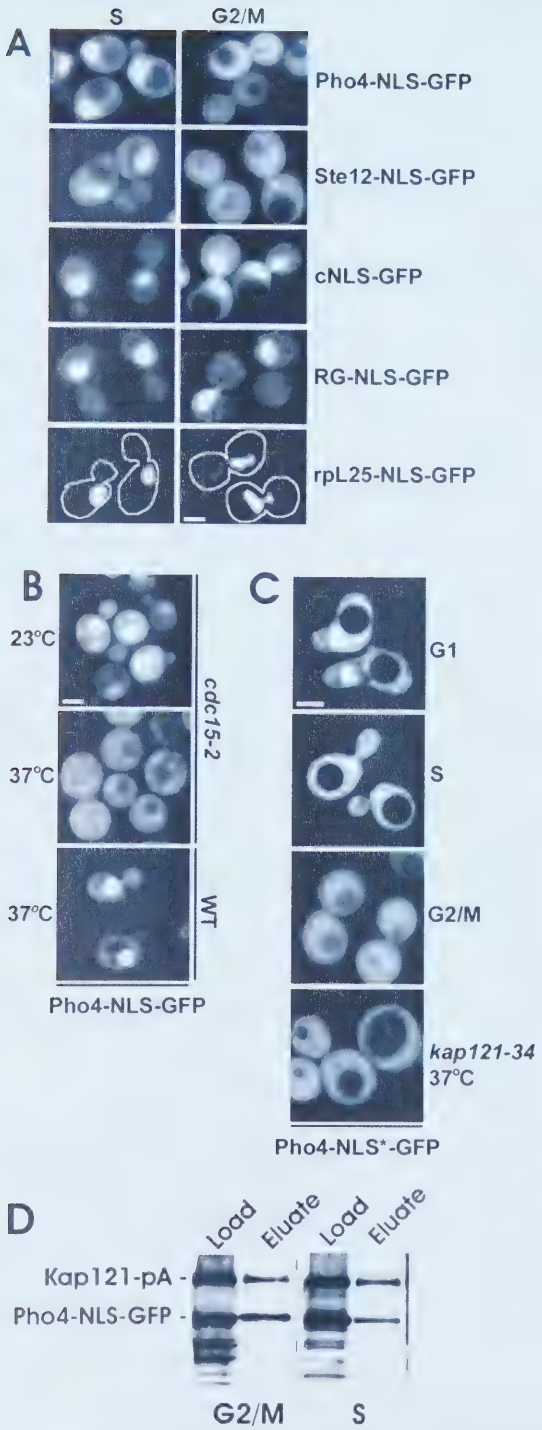

Figure 4-2. Kap121p-mediated import is inhibited during mitosis.

accumulate in the nucleus and were distributed throughout the cell in cultures arrested in G2/M (Fig. 4-2A). This same distribution pattern was also visible in a *cdc15-2* mutant (Hartwell et al., 1971) arrested in telophase at the non-permissive temperature (Fig. 4-2B). For comparison, import was also monitored with various other reporters including those recognized by Kap95p/Kap60p (cNLS-GFP), Kap104p (RG-NLS; Lee and Aitchison, 1999), and Kap123p (rpL25-NLS; Rout et al., 1997), each of which continued to accumulate in nuclei of G2/M arrested cells (Fig. 4-2A).

The inability of Kap121p cargo reporters to accumulate in the nucleus could be caused by a reduced binding to Kap121p. For example, the phosphorylation of Pho4p is responsible for the phosphate ion dependent inhibition of Pho4p binding to Kap121p (Kaffman et al., 1998a). The Pho4-NLS-GFP reporter used here is constitutively imported and not affected by phosphate ion levels; however, the Pho4-NLS does contain several potential phosphorylation sites. To determine whether phosphorylation plays a role in inhibiting its import during mitosis, we constructed an NLS that lacked phosphorylation sites by replacing each of the serine and threonine residues with alanines (Pho4-NLS*). This mutant NLS was functional and its nuclear import was dependent on Kap121p (Fig. 4-2C). When examined in nocodazole arrested cells, the import of Pho4-NLS*-GFP was inhibited (Fig. 4-2C) demonstrating that phosphorylation of the reporter does not play a role in blocking its import. Furthermore, we failed to detect a change in the binding of Kap121p to the Pho4-NLS in G2/M-phase arrested cells, with similar amounts of the Pho4-NLS-GFP reporter being bound to Kap121-pA affinity-purified from cells arrested in G2/M- or S-phase (Fig. 4-2D). These results support the idea that M-phase import inhibition is not caused by the inability of Kap121p to bind its cargo.

We concluded that the M-phase specific defect in Kap121p-mediated import was likely due to an inhibition of the translocation of Kap121p through the NPC. To test this idea, we took advantage of previous reports showing that the treatment of cells with metabolic poisons such as 2-deoxyglucose, reduce cellular levels of Ran-GTP (Schwoebel et al., 2002). Because nuclear Ran-GTP is not required for the translocation of at least certain import kaps through the NPC (Kose et al., 1997; Ribbeck et al., 1999; Englmeier et al., 1999; Ribbeck and Gorlich, 2001; Schwoebel et al., 2002), but it is required for their export back to the cytoplasm, treatment with 2-deoxyglucose can lead to kap accumulation in the nucleus (Schwoebel et al., 2002). With these observations in mind, we specifically monitored the import step of the Kap121p transport cycle by examining the subcellular distribution of Kap121-GFP in G1-, S-, and G2/M-phase arrested cells following treatment with 2-deoxyglucose. Treatment of cells with 2-deoxyglucose rapidly (within 15 min) caused an accumulation of Kap121-GFP in the nucleus of cells arrested in G1-phase (Fig. 4-3A). By comparison, G2/M-arrested cells showed little or no nuclear accumulation of Kap121-GFP during the same time period (Fig. 4-3A). We quantified these results using an approach similar to that previously described by Shulga et al. (1996) for determining relative rates of nuclear transport. Arrested cells were treated for various times with 2-deoxyglucose and scored for the nuclear accumulation of Kap121-GFP. In G1 arrested cells, 2-deoxyglucose treatment induced a rapid nuclear accumulation of Kap121-GFP with 50% of the cells showing a nuclear signal within 13 min (Fig. 4-3B). In comparison, G2/M arrested cells showed a much slower rate of nuclear accumulation requiring 35 min to reach similar levels (Fig. 4-3B). These results suggest that a reduced rate of Kap121p translocation through the

Figure 4-3. Movement of Kap121p into the nucleus is inhibited in G2/M-arrested cells.
(A) DF5 cells containing the plasmid pKAP121-GFP were arrested in G1 with α-factor or G2/M with nocodazole (0 min). Cells were then washed and placed in media lacking glucose and containing 100 mM 2-deoxyglucose. The distribution of Kap121-GFP was documented by fluorescence confocal microscopy at the indicated times. Bar, 5μm. (B) In three independent experiments, 50 cells were scored for nuclear fluorescence at various times after addition of 2-deoxyglucose using a confocal microscope. The percentage of cells showing nuclear fluorescence was plotted against time. (Images in "A" provided by T. Makhnevych; data for "B" provided by myself and T. Makhnevych)

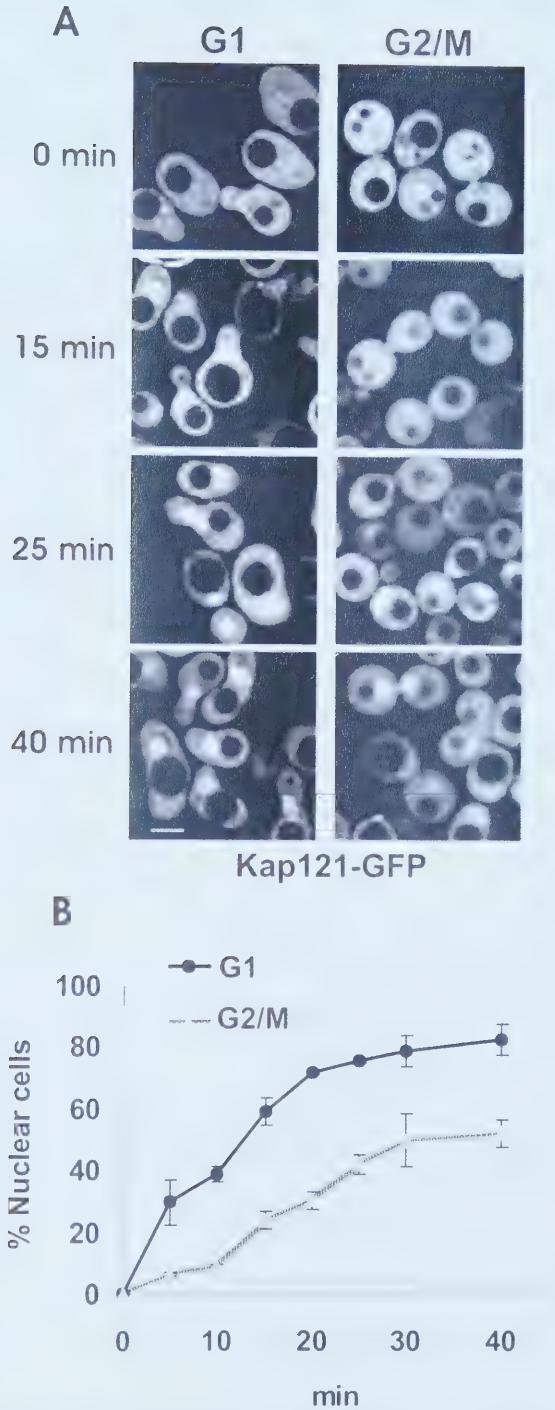

Figure 4-3. Movement of Kap121p into the nucleus is inhibited in G2/M-arrested cells.

NPC accounts for the inhibition of transport during M-phase. Alternatively, exposure to high affinity binding sites could inhibit movement through the translocation channel. Since we did not detect a global change in import, but rather only that mediated by Kap121p, the latter mechanism would likely invoke a nup that interacts specifically with Kap121p. One candidate was Nup53p. Previous work has shown that Nup53p isolated from asynchronous cell cultures is specifically associated with Kap121p (Marelli et al., 1998). However, by the nature of these experiments it was unclear whether this interaction varied during the cell cycle. To address this question, protein A tagged versions of Kap121p or Nup53p were purified from cell cultures arrested in S- or G2/M-phase. Strikingly, these experiments revealed changes in the association of Nup53p with Kap121p during the cell cycle. We failed to detect an interaction between Nup53p and Kap121p in S-phase arrested cells (Fig. 4-4A) following purification of either tagged protein. In contrast, a clear association between these proteins was detected following their purification from G2/M arrested cells (Fig. 4-4A).

The change in the interaction of Nup53p with Kap121p during the cell cycle was accompanied by alterations in its association with neighboring nups. Using affinity purification techniques like those described above, we observed that the association of Nup53p with Nup170p could be detected in S- and G1-arrested cells, but not in cells arrested in G2/M (Fig. 4-4B, see also Iouk et al., 2002). Similarly, we failed to detect an interaction between Nup53p and Nup170p in *cdc15-2* cells arrested at the nonpermissive temperature in telophase (T. Makhnevych and R.W. Wozniak, unpublished). Conversely, Nup53p was tightly bound to purified Nic96p in M-phase arrested cells, but only trace amounts of Nup53p were detected in S-phase arrested cells (Fig. 4-4B). Similar

Figure 4-4. Molecular rearrangements within the NPC during mitosis.
(A,B,C) Cell extracts were prepared from yeast strains producing protein A-tagged Nup53p, Kap121p, Nup170p and Nic96p (NP53pA, KP121pA, NP170pA, and NIC96pA) arrested with hydroxyurea (S) or nocodazole (G2/M). The protein A fusions were affinity purified using IgG-Sepharose beads. The beads were washed and eluted with a step gradient of 200, 500, 1000, and 2000 mM $MgCl_2$. 25% of the eluted proteins were analyzed by SDS-PAGE and Western blotting using α-Nup53p, α-Kap121p, α-Nup57p or α-Nup49p antibodies. Lane L contains a sample of the fraction loaded (0.05%) on the IgG-Sepharose. Contributed by T. Makhnevych.

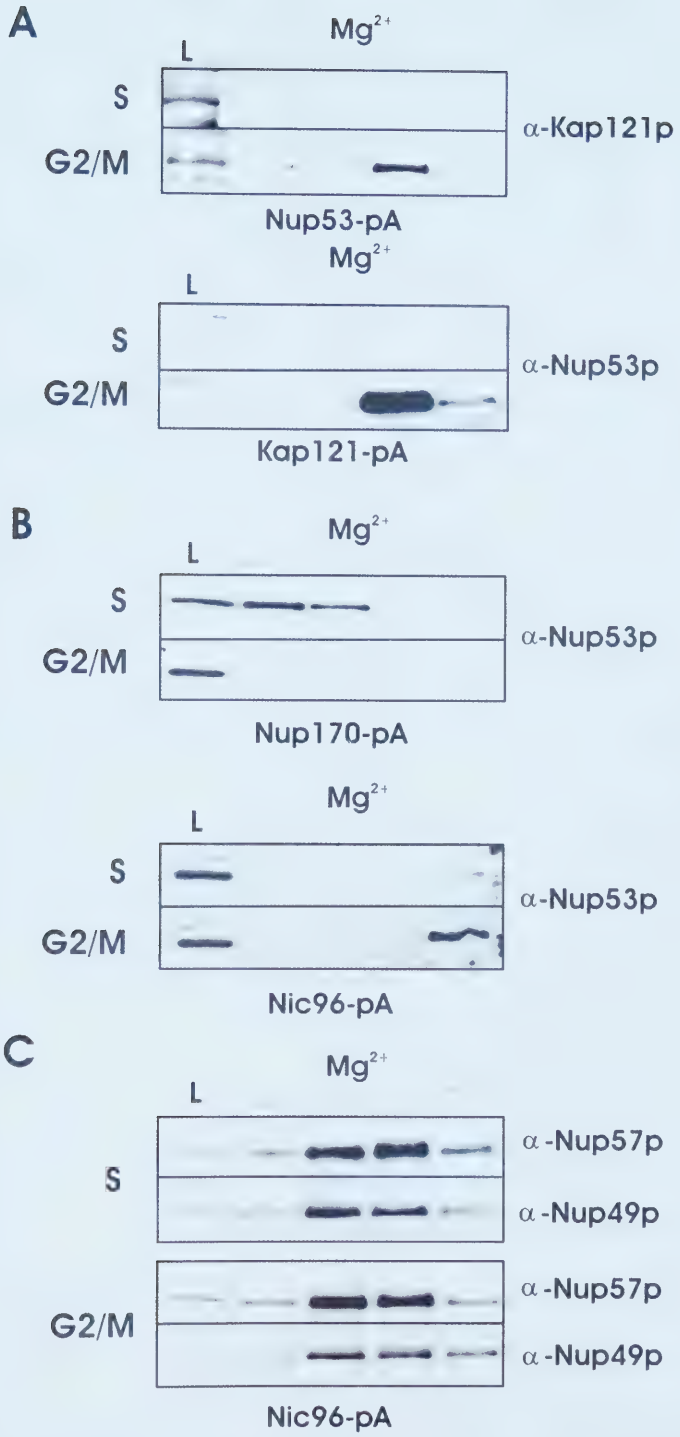

Figure 4-4. Molecular rearrangements within the NPC during mitosis.

molecular rearrangements were not uniformly detected among nucleoporins during the cell cycle. For example, the associations of Nic96p with Nsp1p, Nup57p, and Nup49p were not altered (Fig. 4-4C). These results suggest that specific molecular rearrangements occur in the NPC during M-phase that allow Nup53p to interact with Kap121p and Nic96p.

4.2.3 Nup53p functions to inhibit Kap121p-mediated import during mitosis

The association of Kap121p with Nup53p during M-phase supports the hypothesis that Kap121p-mediated import is inhibited by its interaction with Nup53p. This suggests that mutations in Nup53p that fail to bind Kap121p would increase the rate of transport during M-phase. We therefore examined both the uptake of Kap121p into the nucleus and import of the Pho4-NLS-GFP reporter during M-phase in a strain where Nup53p was replaced with a deletion mutant (*nup53-Δ405-430*) lacking the 26 amino-acid residue Kap121p binding domain (KBD). Cells expressing the *nup53-Δ405-430* mutation were arrested in G2/M-phase and the nuclear accumulation of Kap121p was then induced using 2-deoxyglucose. As shown in Fig. 4-5A and 5B, deletion of the KBD from Nup53p stimulated uptake of Kap121p into the nucleus during mitosis as compared to WT cells. Consistent with these results, removal of the inhibitory effects of the Nup53p KBD also resulted in the steady-state accumulation of the Pho4-NLS-GFP reporter in the nucleus of G2/M synchronized cells (Fig. 4-5C). These results establish a clear role for Nup53p as an inhibitor of Kap121p-mediated import during mitosis.

We have previously shown that the KBD of Nup53p shares sequence similarity with Kap121p-specific NLSs (Fig. 3-4). This similarity suggests the KBD may bind to

Figure 4-5. The KBD of Nup53p is required for Kap121p-mediated import inhibition during mitosis.
(A and B) The localization of Kap121-GFP in DF5 (WT) cells and a *nup53Δ* strain (NP53-Δ405-430) containing a plasmid encoded Nup53p deletion lacking the KBD (nup53-Δ405-430) was examined following G2/M arrest with nocodazole and treatment for 15 min with 100 mM 2-deoxyglucose. The distribution of Kap121-GFP was documented using a confocal microscope. The nuclear uptake of Kap121-GFP was quantified (B) at the indicated times as described in Fig. 4-3B. (C) The distribution of the Pho4-NLS-GFP reporter in the strains NP53-Δ405-430 (nup53-Δ405-430), NP53-FL53 (*nup53Δ* strain containing a plasmid copy of *NUP53*; Nup53p), and DF5 (WT) was examined after G2/M arrest with nocodazole. Bars, 5μm. (D) GST-tagged Nup53p or a truncation of Nup53p lacking the KBD (GST-1-375) were synthesized in and purified from *E. coli.* 5 μg of each fusion was immobilized on GT-Sepharose beads. The beads were then incubated with (+) or without (-) ~10 μg of a pre-formed complex of Kap121p and Pho4p (L). After washing, the bound fraction was eluted with SDS-PAGE sample buffer. Equivalent amounts of the bound and flow through (FT) fractions were analyzed by SDS-PAGE and proteins were visualized by Coomassie blue staining. "A-C" contributed by T. Makhnevych.

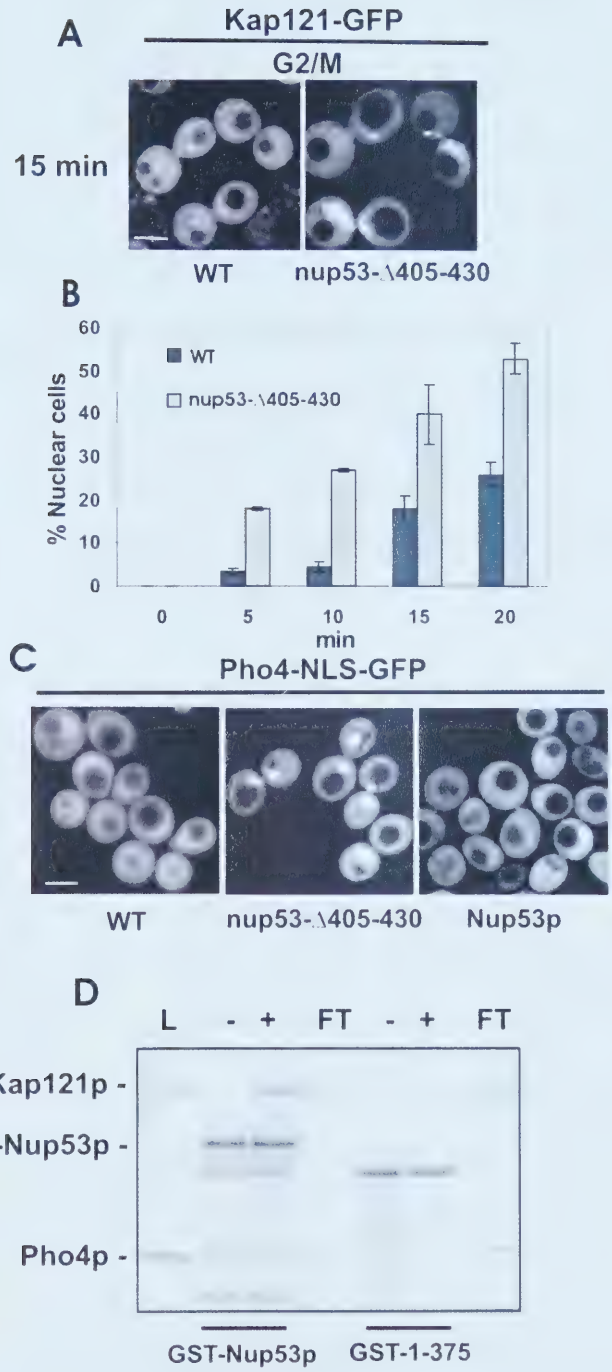

Figure 4-5. The KBD of Nup53p is required for Kap121p-mediated import inhibition during mitosis.

the NLS binding region of Kap121p. This model predicts that the Nup53p KBD may stimulate the release of cargo from Kap121p by competing for NLS binding. To test this, we assembled a recombinant Kap121p/Pho4p complex by incubating Kap121p with immobilized GST-Pho4p. After washing to remove free Kap121p, the Kap121p/Pho4p complex was released from GST (Fig. 4-5D, lane L) and presented to bead-bound GST-Nup53p. We detected that the majority of Kap121p was bound to Nup53p, while Pho4p was detected exclusively in the unbound fraction (Fig. 4-5D, lane FT). In contrast, a deletion mutant of Nup53p lacking the KBD (GST-1-375) failed to alter the Kap121p/Pho4 complex. These results are consistent with a model in which Nup53p also inhibits cargo binding to Kap121p during mitosis.

4.2.4 Increased levels of Nup53p inhibit progression through mitosis

As discussed above, masking of the Nup53p KBD from Kap121p is likely controlled by interactions with Nup170p (Fig. 4-4B; Fig. 3-8). A prediction of this model is that an increase in the levels of Nup53p that exceed the binding capacity of Nup170p would constitutively inhibit import and perhaps cause similar cell cycle defects to those noted in the *kap121-34* ts strain (Fig. 4-1). In fact, increased levels of Nup53p have been shown previously to inhibit import of Kap121p cargo (Marelli et al., 2001). We tested whether moderate increases in the levels of Nup53p would also delay progression through mitosis. For these experiments, *NUP53* under the control of the *CUP1* promoter was introduced into a *nup53Δ* mutant strain. In the absence of induction, this construct produces approximately 5-10 fold higher amounts of Nup53p than the endogenous levels

Figure 4-6. Increased levels of Nup53p delay progression through mitosis.
(A) FACS analysis was performed on logarithmically growing cultures of DF5 (WT) cells and *nup53*Δ strains containing plasmid-encoded *NUP53* (NP53-FL53) or *nup53-Δ405-430* (NP53-Δ405-430) and producing moderately increased levels of Nup53p (↑Nup53p) or nup53-Δ405-430 (↑nup53-Δ405-430), respectively. The positions of 1C and 2C peaks are indicated. (B) The morphologies of cells in these cultures were also examined and the percentage of large budded (LB) cells was compared to the combined percentage of unbudded and small budded (UB/SB) cells. (C) Logarithmically growing cultures of DF5 cells containing an empty plasmid (WT), NP53-FL53 cells (↑Nup53p), NP53-Δ405-430 cells (↑nup53-Δ405-430), and NP53-1-448 (↑nup53-1–448) were arrested with α-factor in G1 (time = 0 min). α-factor was then removed and cells were grown at 23^0C in YPD. Samples were taken at the indicated times (min) and total cell lysates were prepared. Proteins separated by SDS-PAGE and analyzed by Western blotting using antibodies directed against Clb2p (α-Clb2p) and a load control, Gsp1p (α-Gsp1p). Contributed by T. Makhnevych.

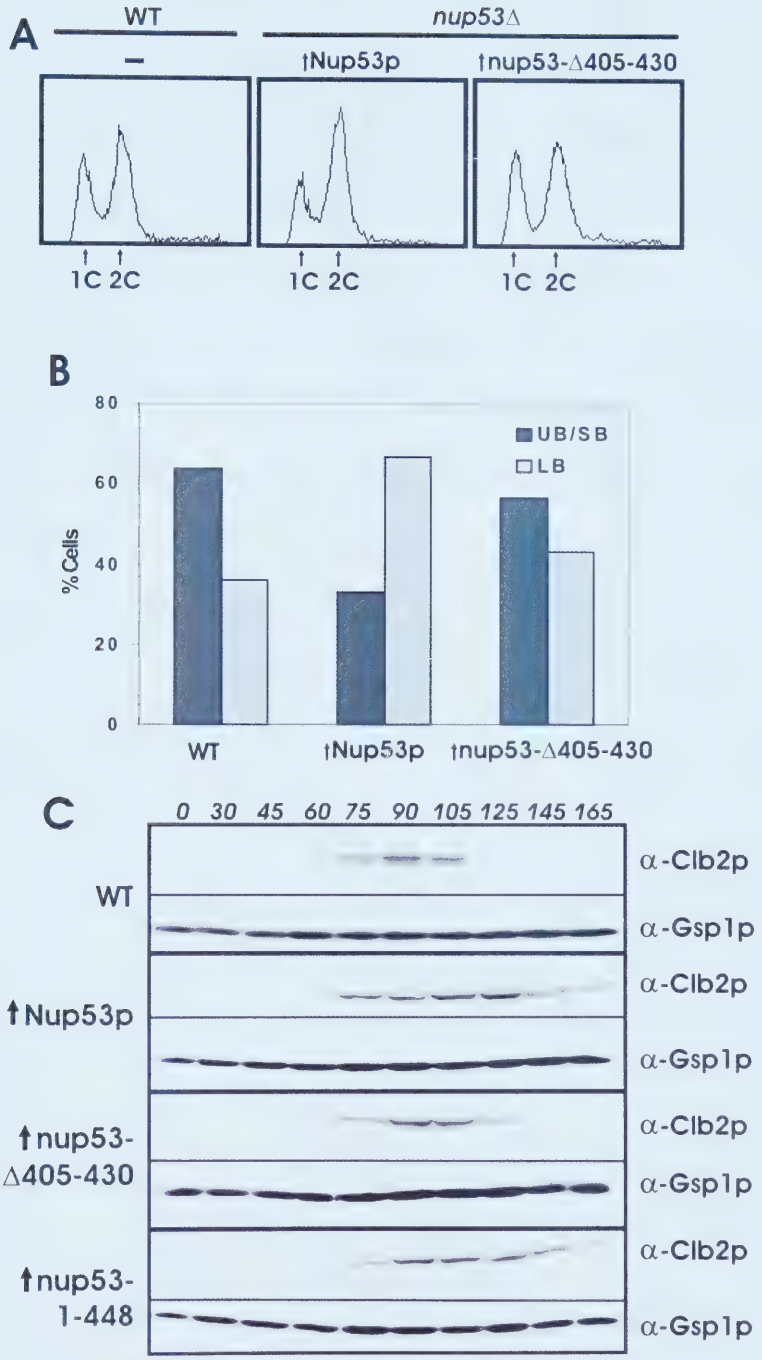

Figure 4-6. Increased levels of Nup53p delay progression through mitosis.

(see Fig. 1C of Marelli et al., 2001). As shown in Fig. 4-6A and 6B, under these conditions of moderate *NUP53* overexpression, the cell population displayed increased numbers of cells containing a 2C complement of DNA and a higher percentage of large-budded cells as compared to a wild-type counterpart. Moreover, a strain overproducing the nup53-Δ405-430 deletion construct lacking the KBD showed a normal distribution of DNA complement and budding index. An examination of Clb2p levels (Fig. 4-6C) in Nup53p overproducing cells also mimicked the *kap121-34* mutant cell cycle phenotypes described above. The delay observed in the degradation of Clb2p was not a function of the inner nuclear membrane proliferation previously observed in cells massively overproducing Nup53p (Marelli et al., 2001), as similar results were obtained upon overproduction of a Nup53p C-terminal truncation that contains the KBD and targets to the NPC, but fails to induce membrane proliferation (nup53-1-448; Fig. 4-6C).

4.3 Discussion

Here we describe a novel mechanism for regulating nuclear import that is controlled by the NPC. While it is generally accepted that the regulation of nucleocytoplasmic transport in response to environmental cues or progression through the cell cycle can be controlled by modifications of a cargo, which alter binding to its cognate karyopherin (see section 1.6.1), our data demonstrate that cell cycle changes in the NPC itself can regulate specific transport pathways. In particular, we show that Nup53p acts as a transport inhibitory nucleoporin (iNup), slowing nuclear transport mediated by Kap121p during mitosis. These results establish that the NPC plays a more elaborate role in mediating nucleocytoplasmic transport than previously appreciated.

Kap121p–mediated import is active in interphase but is inhibited during mitosis. This is in contrast to import mediated by several karyopherins tested here, including the Kap60p/Kap95p complex, Kap123p and Kap104p, which appear unaltered during the cell cycle. A decrease in the steady-state nuclear levels of Kap121p cargo was observed at the G2/M transition (in nocodazole arrested cells, Fig. 4-2) and into telophase (in arrested cdc15-2B cells, Fig. 4-2B). This decreased nuclear accumulation could not be attributed to changes in cargo binding to Kap121p (Fig. 4-2C and 2D) or in the permeability of the NE as measured using diffusion assays (T. Makhnevych and R.W. Wozniak, unpublished). Rather, import inhibition is apparently due to a decrease in the efficiency of Kap121p translocation through the NPC (Fig. 4-3).

An obvious candidate for controlling the altered transport of Kap121p was Nup53p. We have previously shown that Nup53p binds specifically to Kap121p (Marelli et al., 1998) and we identified a region within Nup53p resembling a Kap121p-specific

NLS (Fig. 3-4) responsible for this interaction. This region of Nup53p is contained within a domain that also directly interacts with a neighboring nup, Nup170p. Thus binding to Nup170p prevents Nup53p from interacting with Kap121p (Chapter III). Analysis of this molecular switch revealed the surprising observation that during interphase and active Kap121p transport, most of Nup53p is associated with Nup170p; not Kap121p. Whereas during mitosis, when Kap121p transport is blocked, Nup53p is tightly associated with Kap121p but remains anchored to the NPC through Nic96p. These data suggest the counterintuitive model in which Kap121p binding to Nup53p inhibits transport; rather than promoting it (Fig. 4-7). Indeed, this idea is consistent with previous data showing that *nup53Δ* mutants do not block the nuclear accumulation of Kap121p cargo and that import inhibition can be seen upon overproduction of Nup53p (Marelli et al., 2001), a circumstance that would create a pool of Nup53p free of Nup170p.

The specific events that trigger molecular rearrangements within the NPC are unclear, but Nup53p release from Nup170p presumably frees the Kap121p-binding domain (KBD) allowing Nup53p to bind tightly to Kap121p (Fig. 4-4A), inhibiting transport. This is supported by the striking observation that the removal of the KBD from Nup53p relieves the M-phase specific import inhibition (Fig. 4-5B). Furthermore, it is likely that the phosphorylation of Nup53p (Marelli et al., 1998) and potentially other nups are a key step in this process. At present it is unknown precisely how this modification affects Nup53p's association with its neighboring nups; however, phosphorylation does not appear to influence its binding to Kap121p (Marelli et al., 1998,

Fig. 8-2, and see section 6.4). These data reinforce the idea that the binding of Nup53p to Kap121p during mitosis is controlled by the accessibility of the KBD.

To derive an explanation for how these binding events control Kap121p transport, one must first consider current models for transport through the NPC. It is believed that the kap binding FG-nups, including Nup53p, line the translocation channel through the NPC, forming thousands of binding sites per pore (Rout et al., 2000). Models explaining the movements of kaps through this sea of FG repeat domains (see section 1.5) rely on the assumption that kap-FG-nup interactions must be of low affinity to achieve reasonable rates of translocation through the channel. Indeed, the K_D of kap-FG-nup interactions that have been measured in the micromolar range (Bayliss et al., 1999, Pyhtila and Rexach, 2003). In contrast, NLS interactions with kaps are of higher (nanomolar K_D) affinities (Gilchrist et al., 2002). The structural similarity of the Nup53p KBD to the Kap121p NLS sequence (Fig. 3-4) suggests that this interaction is more similar to a kap-cargo interaction than a kap-FG repeat interaction, and thereby, presumably of higher affinity. Consistent with this idea, it is our empirical observation that the association of Nup53p with Kap121p is stronger than that between Kap121p and other FG-nups, as the latter group can be dissociated from Kap121p under conditions that do not affect the interaction of Nup53p with Kap121p (M. Marelli, T. Makhnevych and R.W. Wozniak, unpublished).

In addition to slowing the rate of Kap121p movement through the NPC during mitosis, our data suggest a second factor contributing to import inhibition is the release of cargo stimulated by competition with Nup53p for the NLS binding site on Kap121p (Fig. 4-5D). Since Nup53p is positioned on both the cytoplasmic and nucleoplasmic faces of

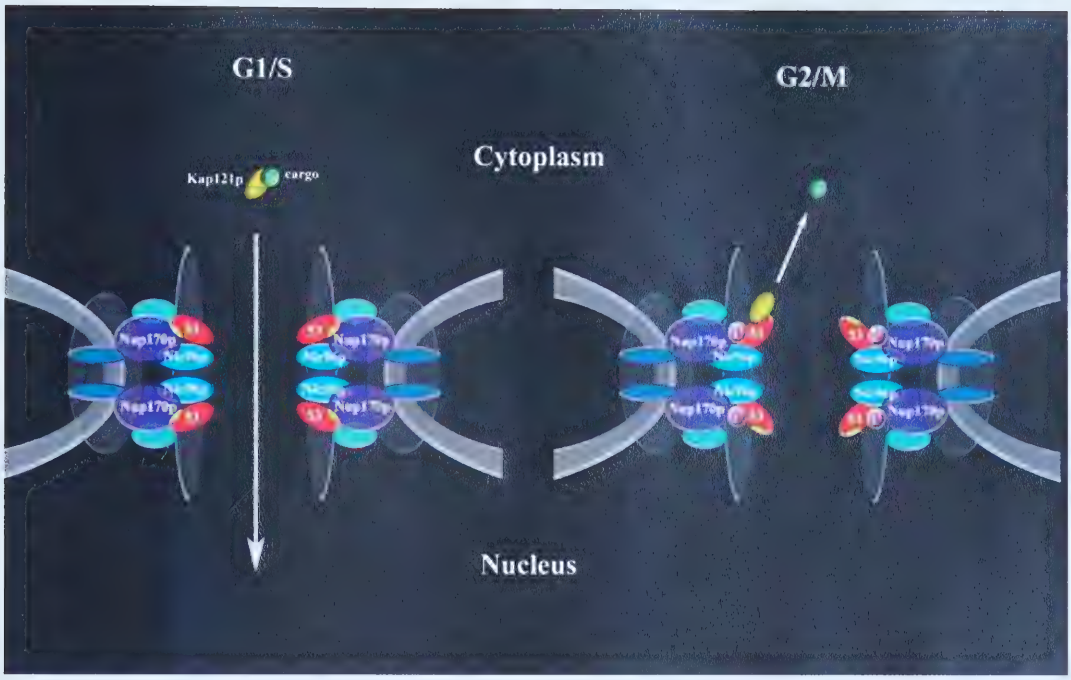

Figure 4-7. A model for the cell cycle regulation of Kap121p-mediated transport by Nup53p.

Kap121p-mediated transport is proposed to proceed through interactions with FG-nups (represented in light blue). During interphase Nup53p is bound to Nup170p, which masks the KBD of Nup53p and prevents its interaction with Kap121p. As cells enter M-phase, Nup53p is phosphorylated (P), no longer bound to Nup170p, and detected in complex with Nic96p. Release from Nup170p exposes the KBD (shown in yellow) and allows Kap121p to bind Nup53p. We propose that the KBD is a high affinity Kap121p binding site, and therefore serves to slow Kap121p's translocation through the NPC. In addition, competition between the KBD and a cargo NLS results in the premature release of cargo from Kap121p.

the NPC (Marelli et al., 1998; Rout et al., 2000), exposure of the Kap121p/cargo complex to cytoplasmically disposed Nup53p could induce cargo release prior to translocation through the NPC (Fig. 4-7). A nup induced cargo release mechanism has also been proposed for the release of cargo from Kapβ2/transportin upon binding to an M9 NLS site in mammalian Nup98 (Fontoura et al., 2000, and see section 1.6.2 for more examples). In this case, however, the localization of Nup98 to the nuclear side of the NPC suggests that this represents a terminal step in the release of cargo into the nucleoplasm. Moreover, both mammalian Nup153 and yeast Nup1p form particularly stable interactions with Kap-β1/importin β (Shah et al., 1998) and Kap95p (Pyhtila and Rexach, 2003), respectively. These nups are also localized exclusively on the nucleoplasmic face of the NPC, which has led to the proposal that they facilitate transport by acting as high affinity sites that draw these kaps away from low affinity FG-sites lining the channel (Ben-Efraim and Gerace, 2001; Pyhtila and Rexach, 2003). Thus, the functions of Nup153, Nup98, and Nup1p as potential facilitators of transport and Nup53p as an iNup may be governed by their localization within the NPC.

Having uncovered a mechanism for regulating the entry of Kap121p into the nucleus during mitosis, we are faced with the question of what role Kap121p-mediated import plays in cell cycle progression. We have shown that reduced import caused by the *kap121* ts alleles or overproduction of Nup53p delays progression through mitosis beginning at or following metaphase (Fig. 4-1 and Fig. 4-6). We surmise that, since levels of Kap121p-mediated import are normally reduced during mitosis (Fig. 4-2), constitutive defects in Kap121p import may produce a mitotic phenotype by interfering with the nuclear influx of cargos occurring at transition points prior to and/or near the end

of mitosis. Multiple Kap121p cargos are likely affected, one of which is Cdh1p, a non essential protein that binds the anaphase promoting complex (APC) and mediates the degradation of Clb2p (see Zachariae and Nasmyth, 1999). Cdh1p is localized to the cytoplasm during mitosis but accumulates in the nucleus late in mitosis where it is detected during G1 (Jaquenoud et al., 2002). The nuclear accumulation of Cdh1p during G1 is dependent on Kap121p and is inhibited in *kap121* ts mutants (Jaquenoud et al., 2002).

The inhibition of Kap121p import during mitosis likely contributes to reducing the nuclear concentration of multiple Kap121p cargo molecules. Which cargos are normally targeted by this regulation, and the specific role that this transport regulation plays in mitosis, however, remain unclear. Defining these functions is complicated by the observations that cells lacking *NUP53* or expressing the *nup53-Δ405-430* deletion, conditions that clearly relieve Kap121p import inhibition (Fig. 4-5), do not exhibit a clear defect in progression through mitosis. These observations are likely explained by two factors, 1) multiple, and often redundant, transport pathways control cargo localization and 2) cargos themselves exhibit redundant functions. The distribution of cargos between the nucleus and the cytoplasm is often controlled by the concerted action of both import and export kaps (Section 1.4), where the steady-state is achieved based on the relative activities of the two pathways. A case in point is again Cdh1p whose localization is controlled by Kap121p (import) and Msn5p/Kap142p (export) (Jaquenoud et al., 2002). We predict that the inhibition of Kap121p import and active export contribute to excluding Cdh1p from the nucleus until the later stages of mitosis.

Several other results also implicate the control of Kap121p import in M-phase progression. We have detected functional interactions between Nup53p and Lte1p, one of a group of proteins functioning in the mitotic exit network (see Jensen and Johnston, 2002), with double null strains exhibiting a heightened cold sensitivity (Fig. 4-8). In addition, we have also detected delays in mitotic progression in strains expressing *nup53-Δ405-430* under conditions of spindle checkpoint activation (Scott et al., 2005). This observation may also help to explain why we have detected a functional interaction between Nup53p and Ctf18p, as strains with mutations in *CTF18* require a functional spindle checkpoint for viability (Fig. 4-8; Hanna et al., 2001). Furthermore, previously unexplained data, including the synthetic lethal interactions of *NUP170* and *SIC1* (an inhibitor of the Cdk1p-Clb2p complex) null mutants and the suppression of a *cdc14* mutant by overexpression of *KAP121* (Yuste-Rojas and Cross, 2000) further underscore a role for the Nup53p transport inhibitory mechanism during mitosis. Fully understanding the functions of this pathway will require defining the repertoire of Kap121p cargos, understanding functional redundancy with other transport pathways, including others linked to mitotic progression (Asakawa and Toh-e, 2002; Shou and Deshaies, 2002), and integrating this information with the known, and often redundant, functions their cargos play in regulating mitosis.

In conclusion, our data demonstrate the existence of a previously undefined mechanism for regulating nucleocytoplasmic transport. This mechanism involves a direct role for the NPC, through cell cycle specific changes in its molecular organization, in regulating specific karyopherin movements and participating in cell-cycle control events. While our experiments have focused on yeast, it seems likely that similar NPC-

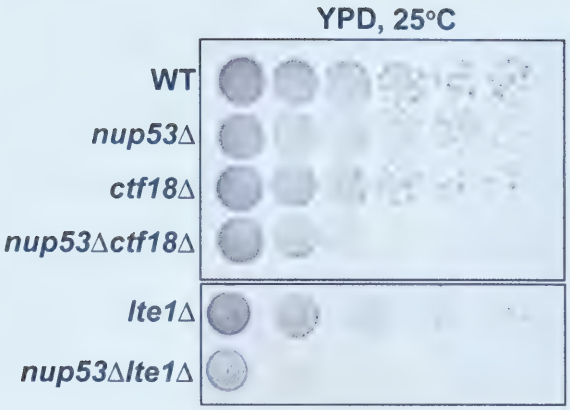

Figure 4-8. Functional interactions between Nup53p, Lte1p and Ctf18p.

A wildtype yeast strain,BY4741 (WT), along with the indicated null strains (nup53Δ, ctf18Δ, lte1Δ, CPL14 and CPL35) were grown to mid-log phase and spotted in 10-fold serial dilutions on YPD plates. Plates were grown for 3 days at 25°C before being imaged.

mediated regulatory events also function in higher eukaryotes, perhaps at points prior to NE disassembly or shortly after its reformation, as well as at other points in the cell cycle. This latter idea is stimulated by previous observations in mammalian cells showing that a subset of nups, including Nup153, Nup214, and Nup358, are phosphorylated during S-phase when the NE is intact (Macaulay et al., 1995; Favreau et al., 1996), as well as data showing that phosphorylation, most likely of nups, can inhibit nuclear transport (Kehlenbach and Gerace, 2000, see section 1.7).

Chapter V: *Nup53p is a target of two mitotic kinases, Cdk1p and Hrr25p*[*]

[*] A similar work will be submitted for publication.

5.1 Overview

In an effort to develop more insight into the molecular mechanisms that drive the mitotic-specific rearrangements of Nup53p and its binding partners, we hypothesized that the phosphorylation of Nup53p may trigger these changes. In order to begin to analyze the potential function of Nup53p phosphorylation, we individually screened 119 of 122 established or predicted yeast kinases for their ability to phosphorylate Nup53p. In this fashion we identified Hrr25p, a casein kinase that distributes to numerous cellular structures including spindle pole bodies, as a major regulator of Nup53p phosphorylation. Hrr25p forms a stable complex with Nup53p *in vitro*, and this interaction appears to be destabilized by phosphorylation. This result may be reflected in the loss of association of Nup53p with Hrr25p *in vivo,* when cells are arrested in mitosis. We also provide evidence that Nup53p is a target of the mitotic kinase Cdk1p/Cdc2p/Cdc28p. The mutation of Cdk1p phosphorylation sites within Nup53p impacts on Nup53p function, as the resulting *nup53* allele cannot fully complement *NUP53* synthetic lethal interactions.

5.2 Results

5.2.1 The phosphorylation of Nup53p

Previous work in our laboratory demonstrated that Nup53p is a target of phosphorylation events in the final stages of mitosis. Specifically, we observed a change in the electrophoretic mobility of Nup53p in cell lysates derived from a yeast strain carrying a temperature sensitive allele of *CDC15*, *cdc15-2*, which at its restrictive temperature arrests in telophase (Hartwell, 1971; Marelli et al., 1998). This change in mobility on SDS-PAGE gels could be reversed by treating the lysates with alkaline phosphatase. As this analysis was preliminary, we wished to further explore the conditions under which we observed Nup53p phosphorylation to gain better insight into the potential functional roles of this modification.

To examine the phosphorylation state of Nup53p, we synchronized cells in G1-phase using α-factor. Following release from arrest, samples were collected from the synchronous cultures as they progressed through the cell cycle. The phosphorylation state of Nup53p was evaluated as changes in mobility on SDS-PAGE gels. The timing of entry and progression through mitosis was evaluated by monitoring levels of the mitotic cyclin Clb2p. Clb2p is a mitotic cyclin which is used as a marker for mitotic entry and exit, but also provides a good indicator of Cdk1p-Clb2p activity. As shown in Fig. 5-1A, Nup53p is visible as a single species at early time points after release from α-factor arrest (0-30 min). By 60 minutes and peaking at 80 minutes an increasing amount of Nup53p exhibited a reduced mobility consistent with an increase in its phosphorylation state. These modified forms of Nup53p then steadily diminished until undetectable at the 120 minute time point. Peak levels of Nup53p phosphorylation appeared to occur at time

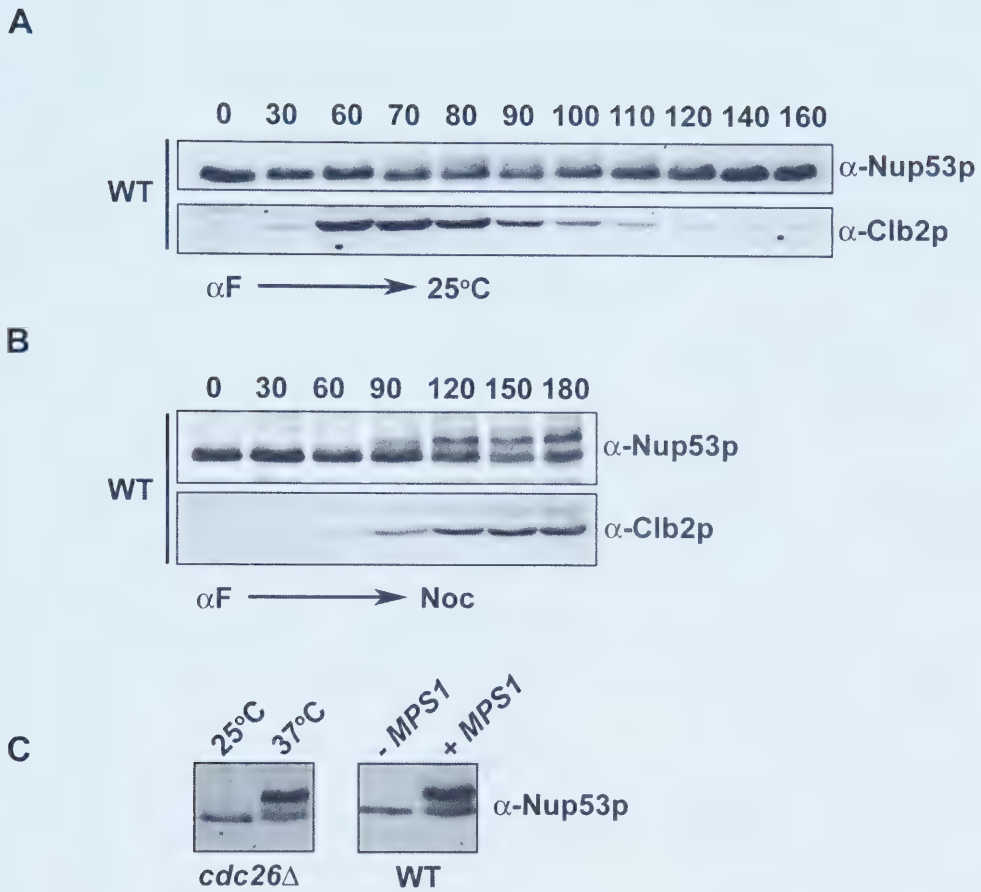

Figure 5-1. The phosphorylation of Nup53p.

The electrophoretic mobility of Nup53p was analyzed in a wild-type (WT) yeast strain, BY4741, synchronized in G1 with α-factor (αF/time 0). The cells were released into (A) media at 25°C, α-factor was re-added at 60 minutes to prevent the initiation of another cell cycle, or (B) into media containing nocodazole (Noc). Samples were collected at the indicated times (min). (C) The phosphorylation of Nup53p was also evaluated in a null strain lacking *CDC26* (cdc26Δ) which arrests in metaphase when grown at 37°C. Samples were collected from mid-log phase cell cultures grown at 25°C and 37°C for 3 hours. Lastly, a yeast strain harboring an extra copy of *MPS1* (CPL61) behind a galactose inducible promoter was grown in glucose *(-MPS1)* or galactose (*+MPS1*) containing media for 6 hours and samples were collected. In all cases, whole cell lysates were prepared from cell samples, and proteins derived from these lysates were separated by SDS-PAGE and deteced by Western blotting. To detect Nup53p and Clb2p, α-Nup53p and α-Clb2p antibodies were used followed by HRP-conjugated secondary antibodies and ECL.

points approximately 10 minutes after maximum amounts of Clb2p were detected. Levels of phosphorylated Nup53p then decreased in parallel with Clb2p degradation. Cycling levels of phosphorylated Nup53p were compared to the phosphorylation state of Nup53p in cells arrested in metaphase by activating the spindle assembly checkpoint (SAC). For these experiments, cells were released from α-factor and arrested in metaphase using the microtubule destabilizing drug nocodazole. Interestingly, we observed significantly higher levels of phosphorylated Nup53p in the metaphase-arrested cells (Fig. 5-1B), than what was observed in cycling cells. By 180 minutes this slower migrating band consisted of at least 50% of Nup53p, and in some cases more (see Fig. 5-2A and 2B). Similar results were also obtained upon activation of the spindle assembly checkpoint by *MPS1* overexpression, and in a temperature sensitive mutant of a component of the anaphase promoting complex (*cdc26Δ*) (Fig. 5-1C).

5.2.2 Cdk1p phosphorylates Nup53p

Our data are consistent with the interpretation that Nup53p phosphorylation is restricted to G2/M-phase, suggesting that kinases active during this period are responsible for these modifications. Support for this idea also comes from a recent proteomic analysis aimed at identifying novel Cdk1p substrates (Ubersax et al., 2003). These studies showed that Nup53p could be a target of the Cdk1p/Clb2p kinase complex *in vitro*. To assess whether Nup53p was a target of Cdk1p phosphorylation *in vivo* we took advantage of a mutant of *CDK1*, *cdk1-as1*, that can be rapidly and specifically inhibited by the addition of an ATP analogue inhibitor (1 NM PPI) to the growth media (Bishop et

al., 2002). Addition of this inhibitor has been shown to effectively abolish cdk1-as1 activity within 5 to 10 minutes. To examine the effects of cdk1-as1 on Nup53p phosphorylation, we arrested both wild-type and *cdk1-as1* strains in G2/M with nocodazole to induce the phosphorylation of Nup53p. Under these conditions, we observed a similar mass shift of Nup53p in both strains, confirming that they were arrested in metaphase (Fig. 5-2A). We then added 1 NM PPI and analyzed samples by Western blotting at 10 and 20 minute time points. As expected, the addition of 1 NM PPI had no effect on the phosphorylation state of Nup53p in wild-type cells (Fig. 5-2A). In contrast, cells harboring the *cdk1-as1* allele exhibited a clear reduction in the amount of phosphorylated Nup53p. We were unable, however, to completely abolish the phosphorylation of Nup53p, even after 20 minutes in the presence of 1 NM PPI.

We recognized that Cdk1p phosphorylates the serine or threonine residue in the consensus sequence S/T-P-x-K/R (Ubersax et al., 2003). Two serine residues (amino acid residues 101 and 206) in Nup53p conform to this consensus. Using site-directed mutagenesis, we altered the codons for these amino acid residues to encode alanine residues (*nup53-2A*). To insure that nup53-2A was produced at similar levels as the wild-type protein, we replaced the endogenous *NUP53* locus with the ORF encoding *nup53-2A*. By Western blotting, we confirmed that nup53-2A was produced at similar levels and stability as Nup53p (Fig. 5-2B). To examine the phosphorylation state of nup53-2A, we synchronized both wild-type and *nup53-2A* cells in G1 using α-factor and released them into media containing nocodazole. We observed that slower mobility forms of Nup53p and nup53-2A began to appear at 90 minutes after G1-release and persisted for the duration of the time course (Fig. 5-2C). The slower mobility form of nup53-2A,

Figure 5-2. Cdk1p phosphorylates Nup53p.
(A) A wild-type (*CDK1*/DOM0090) yeast strain and a strain where the *CDK1* gene has been replaced by an allele encoding *cdk1-as1* (DOM0039) were arrested in G2/M with the addition of nocodazole (Noc) to the growth medium. cdk1-as1 activity can be inhibited by the addition of a chemical ATP analogue, 1 NM PPI. 1NM PPI was added (+) or mock added (-) at a final concentration of 10 μg/ml to both cell cultures and samples were collected after 10 and 20 minutes. (B) A wild-type (*NUP53*/CPL37) yeast strain and a strain where the gene locus encoding *NUP53* was replaced by *nup53-2A* (CPL66), a *nup53* allele in which the codons encoding serine 101 and 206 have been mutated to encode alanine residues, were either grown to mid-log phase and samples were collected or (C) synchronized in G1 (αF) and released into media containing nocodazole (Noc) and sampled at the indicated times. Nup53p and Clb2p were detected by Western blotting as described in Fig. 5-1 legend.

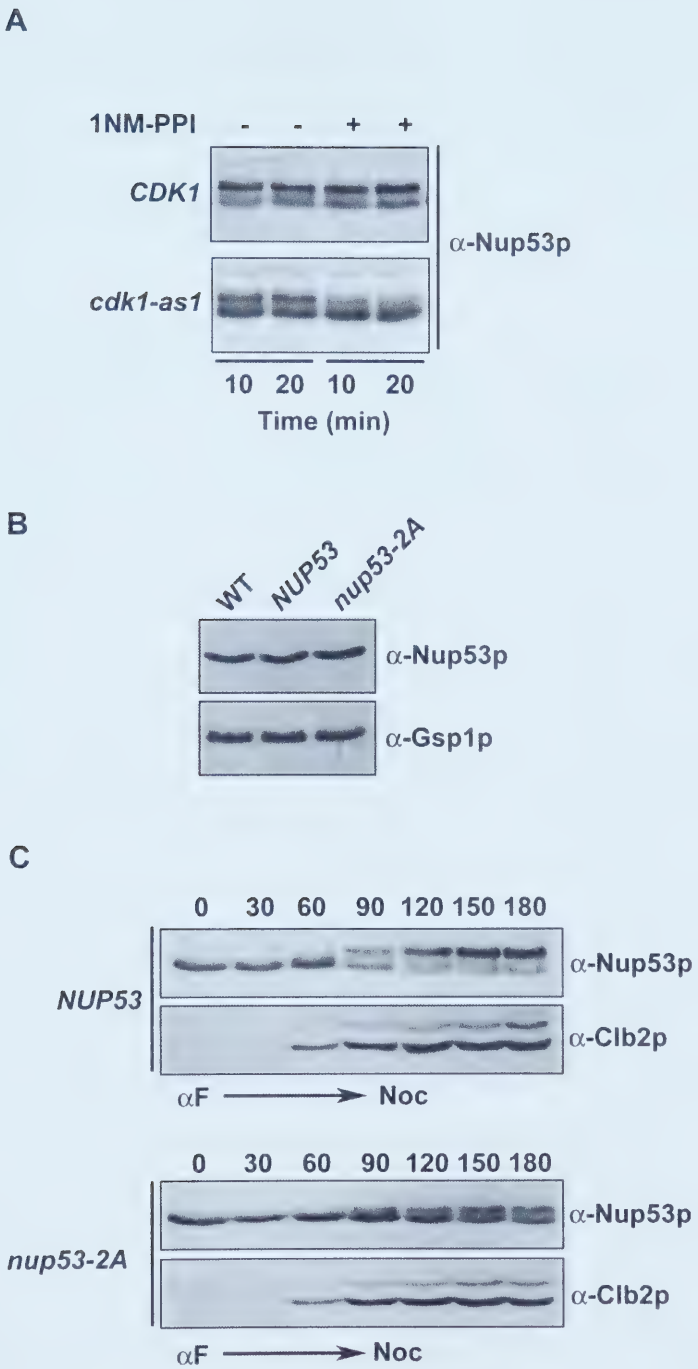

Figure 5-2. Cdk1p phosphorylates Nup53p.

however, was migrating faster than its Nup53p counterpart. Moreover, while the majority of Nup53p was phosphorylated in wild-type cells, only ~50% of nup53-2A was modified. On the basis of these data we suggest that Cdk1p is, in part, responsible for the metaphase-specific phosphorylation of Nup53p.

5.2.3 A screen for a Nup53p kinase

Since we were unable to completely abrogate the phosphorylation of Nup53p by inhibiting cdk1-as1 or by mutating Cdk1p consensus sites in Nup53p, we investigated whether other kinases might contribute to the phosphorylation of Nup53p. To test this idea, we took advantage of a library of GST-tagged kinases compiled by Zhu et al (2000), that are expressed and affinity purified from yeast extracts. Using this library, we individually screened 119 of the 122 established or predicted kinases encoded by the yeast genome for their ability to incorporate $\gamma^{32}P$ into recombinant Nup53p. A portion of an autoradiograph showing 12 reaction products from an initial screen is shown in Fig. 5-3A. Included in this group are the phosphorylated products of the kinase Hrr25p. In addition to Hrr25p, 8 separate kinases were capable of incorporating $\gamma^{32}P$ into Nup53p (Fig. 5-3B). This group of kinases included the remaining 3 members of the casein kinase family (Yck1-3p) as well as Fus3p, Ste20p, Hal5p, Tpk1p and Ptk2p.

With the exception of Hrr25p, none of the kinases capable of phosphorylating Nup53p are essential for cell viability. Thus we were able to test whether the phosphorylation of Nup53p was altered in null strains lacking each of these kinases. In each case, no significant change in the phosphorylation state of Nup53p was observed in

cells arrested in G2/M with nocodazole (Fig. 5-4A), suggesting these kinases are not required for Nup53p phosphorylation *in vivo*.

Nup53p is also a target of the casein kinase Hrr25p. To evaluate this one remaining candidate in phosphorylating Nup53p, we used a conditional allele of *HRR25*, *HRR25*^{DEGRON} (Kafadar et al., 2003). *HRR25*^{DEGRON} encodes an in-frame N-terminal Degron cassette which targets Hrr25p^{Degron} for degradation by the proteasome (Bachmair et al., 1986; Kafadar et al., 2003). The expression of *HRR25*^{DEGRON} is controlled by a galactose-inducible promoter and can therefore be induced or repressed by growing cells in galactose or glucose, respectively. Repressing *HRR25*^{DEGRON} expression in a strain lacking endogenous *HRR25* (KKY387) results in the clearing of all detectable Hrr25p^{Degron} within 5 hours (Kafadar et al., 2003). KKY387 along with its isogenic wild-type strain were grown in media containing galactose as the sole carbon source and synchronized in G1-phase using α-factor. To repress the expression of *HRR25*^{DEGRON}, arrested cells were transferred to glucose containing media. Subsequently, cells were released from α-factor arrest into media containing glucose and nocodazole. Under these conditions the phosphorylation of Nup53p in wild-type cells was detected at 150 minutes following the addition of nocodazole (Fig. 5-4B). In contrast, the phosphorylation of Nup53p in the KKY387 (*HRR25*^{DEGRON}) strain was severely inhibited. Importantly, this attenuation in phosphorylation was not likely the result of the inability of KKY387 to enter into mitosis as Clb2p levels rose at similar times and to similar levels in both the KKY387 and wild-type cells (Fig. 5-4B). Both these data, and the results from the *in vitro* kinase screen, strongly support a role for Hrr25p in regulating the phosphorylation of Nup53p.

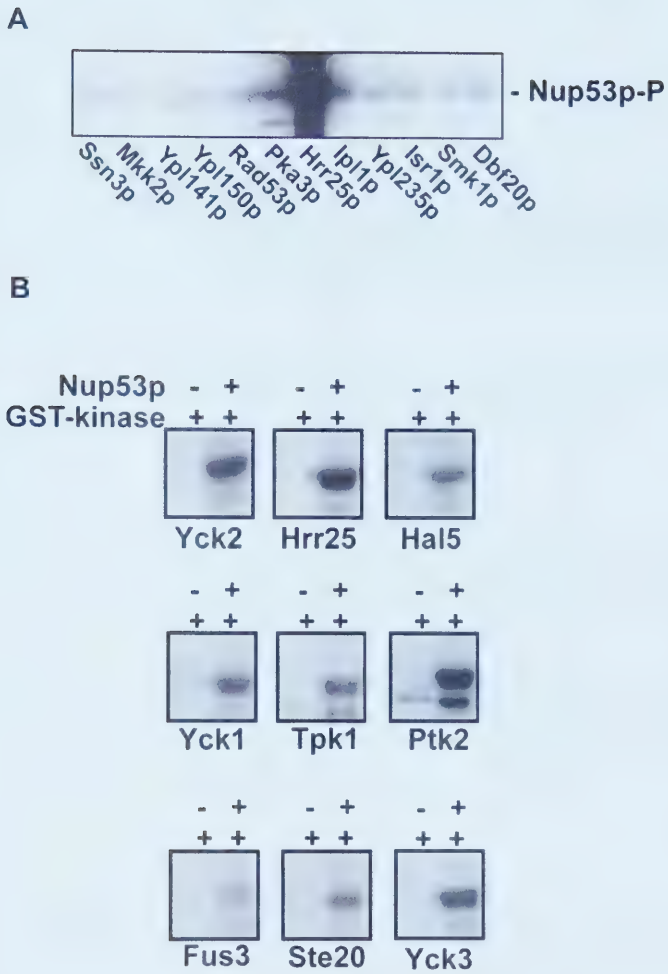

Figure 5-3. A GST-kinase screen.

119 GST-tagged yeast kinases (GST-kinase) were purified from yeast extracts and individually incubated with recombinant Nup53p, and ^{32}P-γ-ATP. Proteins in the reactions were solubilized in SDS-sample buffer, separated by SDS-PAGE and the resulting gels exposed to autoradiographic films. A representative portion of an autoradiograph showing the results of 12 different kinase reactions is shown in (A) Nup53p-P shows the position of phosphorylated Nup53p. (B) Positives were re-screened to better resolve Nup53p in reactions mock incubated (-) or incubated with recombinant Nup53p (+). Contributed by D. Waller.

Figure 5-4. Hrr25p is required for Nup53p phosphorylation *in vivo*

(A) The phosphorylation of Nup53p was monitored in BY4742, a wild-type (WT) yeast strain, and in the indicated null strains grown to mid log phase. Cells were untreated (-) or treated (+) with nocodazole for an arrest in G2/M. (B) A wild-type (YPH499) yeast strain and an *hrr25Δ* null strain covered with a plasmid-encoded conditional allele of *HRR25, HRR25*^{DEGRON} (KKY387) were grown in galactose (Gal) containing media and arrested in G1 using α-factor (αF). To deplete Hrr25p^{Degron}, the cells were resuspended in glucose (Glc) containing media containing α-factor for an additional 2 h. The cells were then released into media containing glucose and nocodazole (Noc). Nup53p and Clb2p were monitored at the indicated time points (min) as described in the legend for Fig. 5-1.

5.2.4 The phosphorylation of Nup53p destabilizes its interaction with Hrr25p

Our data, however, could not exclude the possibility that Hrr25p was indirectly functioning to phosphorylate Nup53p, perhaps by modulating the activity of another kinase. To address this question, recombinant GST-fusions of both Hrr25p and hrr25-K38A, a catalytically inactive mutant of Hrr25p were synthesized in *E. coli*. We then directly tested whether Hrr25p could phosphorylate Nup53p by incubating GST-Hrr25p or GST-hrr25-K38A with recombinant Nup53p and ^{32}P-γ-ATP. As shown in Fig. 5-5A, GST-Hrr25p could phosphorylate Nup53p, while negligible levels (<5% of GST-Hrr25p) of Nup53p phosphorylation were detected with GST-hrr25-K38A.

Our observation that Hrr25p could directly phosphorylate Nup53p prompted us to examine whether these proteins were capable of forming a stable complex *in vitro*. To test this, GST-Hrr25p or GST alone were incubated with purified recombinant Nup53p at 4°C in the absence of ATP. The results of these experiments showed that Nup53p bound to GST-Hrr25p, but not to GST alone (Fig. 5-5B). As these experiments were performed in the absence of ATP, it was unlikely that the phosphorylation of Nup53p was required for its binding to GST-Hrr25p. Conversely, it is possible that the phosphorylation of Nup53p played a role in destabilizing its interaction with Hrr25p. To test this idea, preformed complexes of GST-Hrr25p-Nup53p were incubated with ATP or buffer alone and the release of Nup53p was monitored. As shown in Fig. 5-5C and D, ATP addition induced the release of ~3 fold more Nup53p from GST-Hrr25p, than incubation with buffer alone.

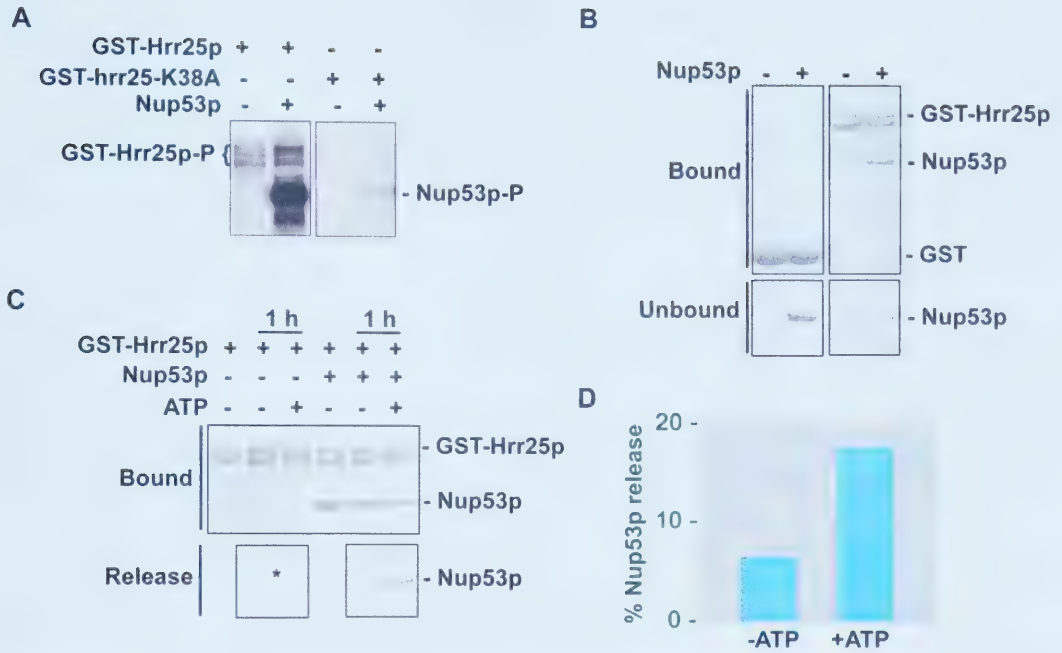

Figure 5-5. Hrr25p directly phosphorylates Nup53p.

Recombinant GST-fusions of Hrr25p, hrr25-K38A and GST alone were immobilized on GT-Sepharose. (A) Bead bound GST-Hrr25p and GST-hrr25-K38A were incubated (+) or mock incubated (-) with recombinant, purified Nup53p and ^{32}P-γ-ATP. After a 1 h incubation, reaction products were separated by SDS-PAGE and the resulting gels were exposed to autoradiographic films. GST-Hrr25p-P indicates the position of phosphorylated GST-Hrr25p (autophosphorylation) and Nup53p-P of phosphorylated Nup53p. (B) Bead bound GST or GST-Hrr25p was incubated with Nup53p. The unbound fraction was collected. After washing the column, proteins were eluted with SDS-PAGE sample buffer and proteins from both bound and unbound fractions were resolved by SDS-PAGE and visualized with Coomassie blue. (C) Either bead bound GST-Hrr25p or a preformed complex of GST-Hrr25p and Nup53p, were split into 3 equivalent aliquots. Proteins from one aliquot were immediately eluted with SDS-PAGE sample buffer (lanes 1 and 4) and the other two were either incubated (+) or mock incubated (-) with 1 mM ATP for 1 h at 30°C. After collecting the "release" fraction, both bound and release fractions were visualized as described in (B) ~70% of the bound and 100% of the release fractions are shown. "*" is an *E. coli* protein that binds to GST-Hrr25p. (D) Release fractions from lanes 5 (-ATP) and 6 (+ATP) were quantitated by a densitometric analysis and displayed graphically as the % of the total Nup53p bound in lane 4. Before calculating the % Nup53p release, the amount of the *E.coli* protein (*) released in Lane 3 was subtracted from the release fraction in Lane 6.

5.2.5 Nup53p interacts with Hrr25p *in vivo*

In an effort to determine whether a similar interaction between Hrr25p and Nup53p exists *in vivo*, we expressed an HA-tagged copy of *HRR25* (*HA-HRR25*, Murakami et al., 1999) in a yeast strain synthesizing a protein A fusion of Nup53p (NP53pA). Whole cell lysates from both asynchronous and nocodazole-arrested cell cultures were prepared and Nup53-pA was purified using IgG-sepharose beads. Proteins bound to Nup53-pA were eluted and analyzed by Western blotting with α-HA antibodies to detect HA-Hrr25p. As shown in Fig. 5-6, HA-Hrr25p was detected in eluted fractions derived from asynchronous cell cultures, but not those of G2/M-arrested cells. While Hrr25p was not detected in G2/M arrested cultures, we did observe the previously described G2/M-specific interaction between Nup53p and the karyopherin Kap121p (Chapter IV).

5.2.6 The subcellular distribution of Hrr25p

Both our *in vitro* and *in vivo* data suggested that Hrr25p and Nup53p directly interact, and that this interaction may be cell cycle regulated. These data are consistent with a model in which the subcellular distribution of Hrr25p dynamically changes during the cell cycle. To evaluate the subcellular localization of Hrr25p, we chromosomally integrated the *GFP* ORF in frame at the 3' end of the endogenous *HRR25* gene locus. Hrr25-GFP was stably produced and the resulting strain had no observable growth defects suggesting that Hrr25-GFP was functional (Fig. 5-7A). Using confocal microscopy, we examined the localization of Hrr25-GFP throughout the cell cycle by

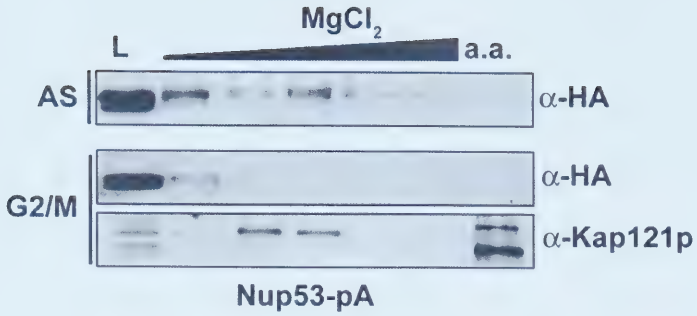

Figure 5-6. Hrr25p interacts with Nup53p *in vivo.*

Cell extracts were prepared from a yeast strain producing protein A-tagged Nup53p (NP53pA) and HA-tagged Hrr25p, from mid-log phase (AS) cells or from those arrested in G2/M with the addition of benomyl to the growth media. Nup53-pA was affinity purified from the cell extracts using IgG-Sepharose beads. The beads were washed and eluted with a step gradient of 50, 200, 500, 1000 and 2000 mM $MgCl_2$ followed by a final elution with acetic acid, pH 3.4. Elutions were precipitated with the addition of trichloracetic acid, resolubilized in SDS-PAGE sample buffer and 50% of the eluted proteins were separated by SDS-PAGE and Western blotted using α-HA and α-Kap121p antibodies, followed by ECL. Lane L contains a sample (0.05%) of the fraction loaded on the IgG-Sepharose and a.a. represents 10% of the acetic acid elution. Contributed by T. Makhnevych.

synchronizing cells in G1-phase using α-factor and then releasing them into media alone, or into media containing nocodazole. In G1-arrested cultures (time 0), Hrr25-GFP appeared to be predominantly concentrated within the nucleus (Fig. 5-7B). As cells progressed out of G1-phase, Hrr25-GFP began to appear at a focus along the nuclear surface which colocalized with the SPB marker protein Spc42-RFP (Fig. 5-7C). At the same time, Hrr25-GFP is transiently visible at the emerging bud neck (30 min). By 60 min, Hrr25-GFP is detected along the bud cortex and is still visualized at SPBs. By 90 min, the cortical localization of Hrr25-GFP is no longer visible and the protein appears predominantly localized to SPBs. The large-budded morphology of these cells, as well as the increased levels of Clb2p detected in this strain background at this time point (Fig. 5-1A) suggest that these cells are in M-phase. Consistent with this idea, in nocodazole-arrested cells Hrr25-GFP is also detected predominantly at one SPB (Fig. 5-7B and C). By later stages of cell division, Hrr25-GFP appears to concentrate at the septin ring, prior to re-accumulation in the nucleus by G1-phase.

5.2.7 *nup53-2A* does not efficiently complement a *nup53Δnup170Δ* strain

In the aggregate, our data are consistent with the mitotic-specific phosphorylation of Nup53p being controlled by two kinases, Cdk1p and Hrr25p. To begin to address the functional role of Nup53p phosphorylation, we evaluated the potential phenotypes associated with the nup53-2A Cdk1p site mutants. As a preliminary step, we tested

Figure 5-7. Subcellular distribution of Hrr25-GFP.
(A) A yeast strain producing a GFP fusion of Hrr25p (CPL6) expressed from its endogenous promoter, was grown to mid-log phase along with its wild-type (WT) parent (BY4741) and spotted in 10-fold dilutions on YPD. The plates were incubated for 48 hours at 30°C before being imaged. (B) CPL6 was synchronized in G1 using α-factor (time 0), and released into fresh media with or without nocodazole (Noc). Images of Hrr25-GFP were acquired by fluorescence confocal microscopy. (C) In an attempt to colocalize Hrr25p with spindle pole bodies, a yeast strain producing Hrr25-GFP and an RFP fusion of a spindle pole body protein, Spc42p (CPL69), expressed from its endogenous promoter was arrested in G2/M with nocodazole. Images of Hrr25-GFP and Spc42-RFP were acquired and "Merge" shows an overlay of these images. Bar, 0.5 μm.

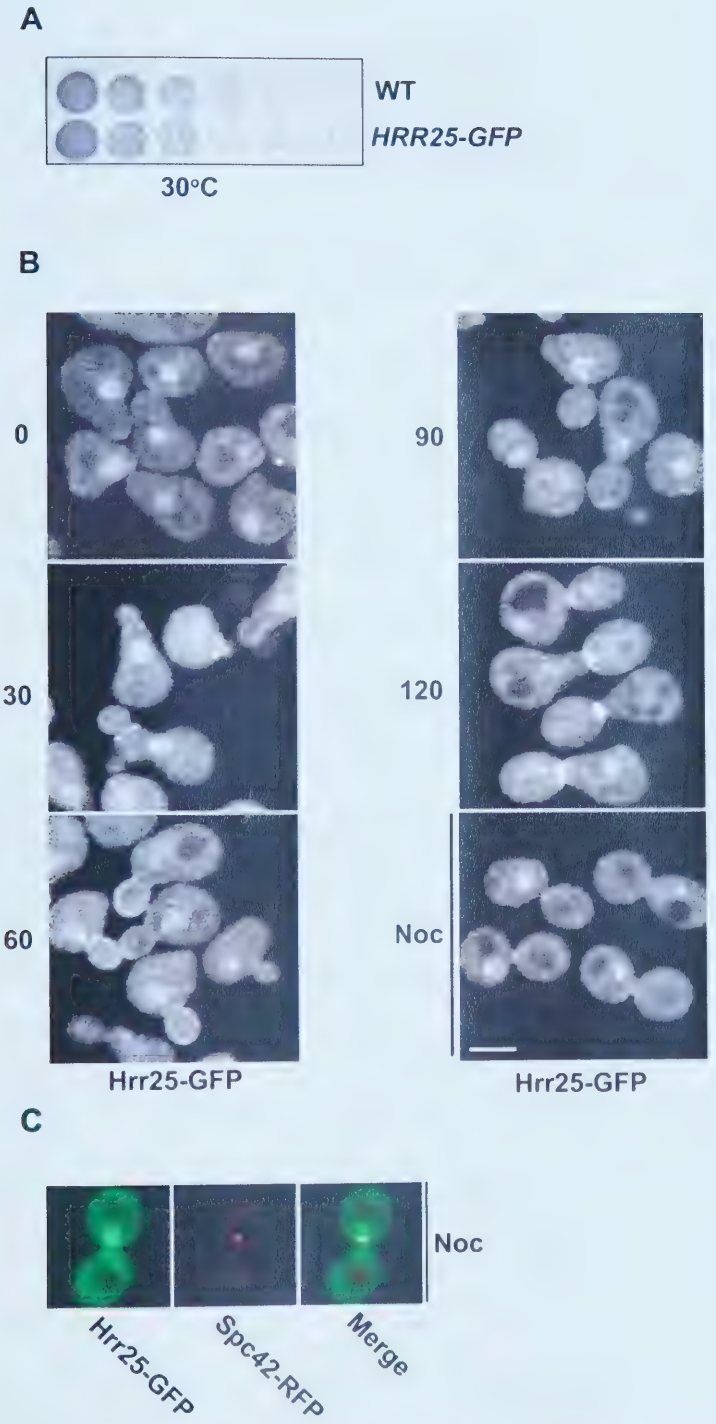

Figure 5-7. The subcellular distribution of Hrr25-GFP

whether strains expressing *nup53-2A* exhibited any growth abnormalities at different temperatures (Fig. 5-8A and B). In addition, since *nup53Δ* strains exhibit a resistance to growth in the presence of the microtubule destabilizing drug, benomyl, (Iouk et al., 2002; Scott et al., 2005), we also tested the growth of *nup53-2A* containing strains on plate media containing this drug (Fig. 5-8B). We were unable, however, to detect any differences between the relative growth characteristics of wild-type *(NUP53)* or *nup53-2A* containing strains (Fig. 5-8A and B). We therefore tested the ability of *nup53-2A* to functionally complement the synthetic lethality of a yeast strain with deletions of both *NUP53* and *NUP170* genes (Marelli et al., 1998). Of a number of known *nup53Δ* synthetic lethal interactions (see Marelli et al., 1998), we chose *nup170Δ* because of its established functional links with cell cycle proteins, including the Cdk1p inhibitor, Sic1p (Yuste-Rojas and Cross, 2000) and the spindle checkpoint protein, Mad1p (Iouk et al., 2002). Furthermore, *nup170Δ* strains exhibit a growth resistance to benomyl similar to that of *nup53Δ* strains, which is thought to be linked to a potential function in the spindle assembly checkpoint (Iouk et al., 2002; Scott et al., 2005). As shown in Fig. 5-8A, *nup53-2A* was unable to complement a *nup53Δnup170Δ* strain as efficiently as *NUP53,* particularly at elevated temperatures. We also observed that *nup53-2Anup170Δ* exhibited a striking growth sensitivity on plate media containing benomyl (Fig. 5-8C).

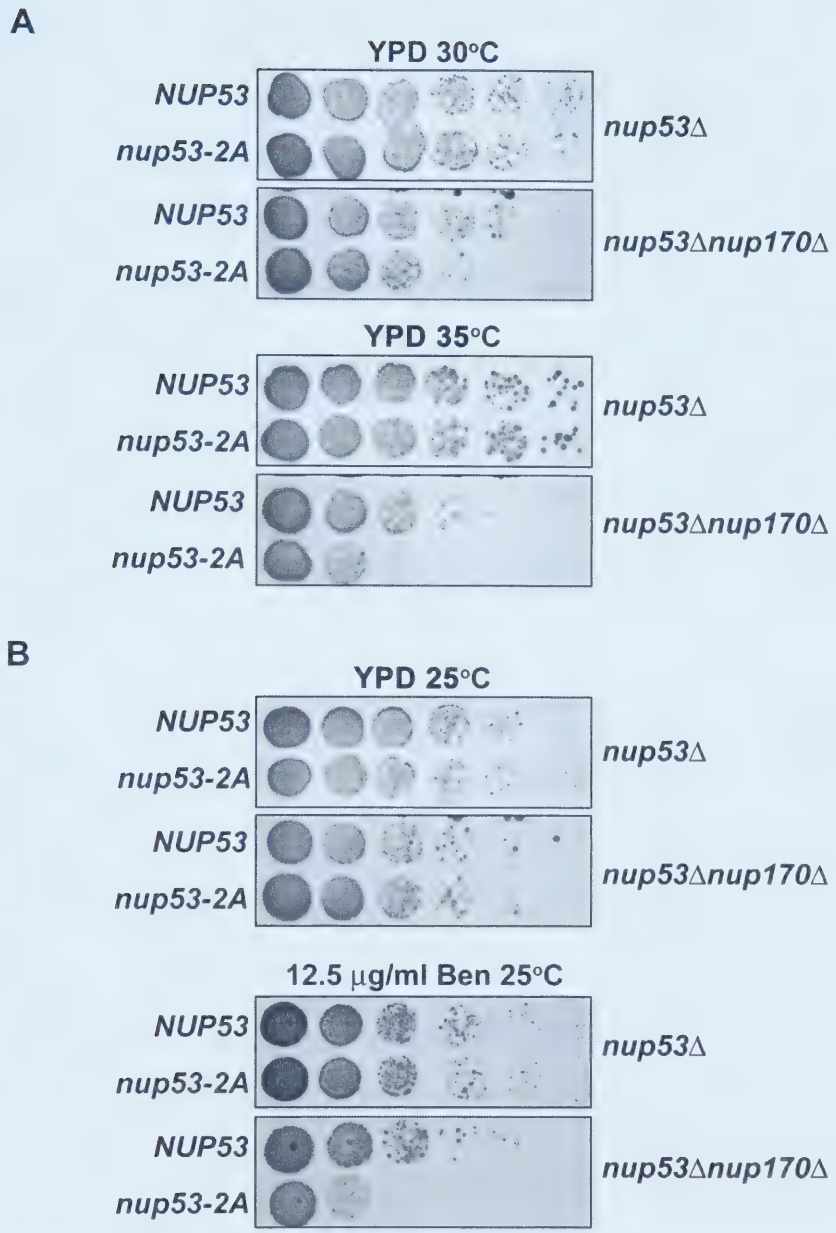

Figure 5-8. *nup53-2A* does not complement a *nup53Δnup170Δ* yeast strain.

(A) *nup53Δ* and *nup53Δnup170Δ* yeast strains transformed with plasmids encoding *NUP53* (p315-NUP53/CPL71/73) or *nup53-2A* (p315-nup53-2A/CPL72/74) were spotted in 10 fold serial dilutions on YPD, and on YPD containing 12.5 µg/ml benomyl (Ben). Images were acquired after growth at the indicated temperatures for 2-5 days.

5.3 Discussion

A number of nucleoporins are modified by phosphorylation, particularly during mitosis, yet the functional significances of these events remain ill defined. This, in part, is a result of the fact that very little is known about the kinases involved in phosphorylating nups, or the amino acid targets of these kinases. Here, we have provided evidence that at least two kinases phosphorylate Nup53p during mitosis in yeast. The first, Cdk1p, is a member of the cyclin dependent kinase family and is a principal regulator of cell cycle progression in all eukaryotes (Morgan, 1997; Nigg, 2001). The second, Hrr25p, is one of four yeast orthologues of the highly conserved casein kinase I family (Gross and Anderson, 1998).

We identified Nup53p as a target of Hrr25p using a kinase screen developed by Zhu et al. 2000, which has been successfully used to identify kinases for other substrates (Kafadar et al., 2003). Out of the 9 kinases that phosphorylated Nup53p *in vitro*, we focussed on Hrr25p primarily because only Hrr25p appeared to influence the phosphorylation of Nup53p *in vivo* (Fig. 5-4). These data alone, however, do not allow for the exclusion of the possibility that other kinases, including those that are functionally related to the remaining 8, might also contribute to Nup53p phosphorylation. In addition, it remains a possibility that certain GST-kinases were not completely functional in the initial screen. For example, while Tpk1p (a cAMP dependent protein kinase) was able to phosphorylate Nup53p, we were unable to detect phosphorylation by other related cAMP dependent protein kinases, including Tpk2p and Tpk3p. This could suggest that Tpk1p can specifically phosphorylate Nup53p, or it is possible that the activity of Tpk2p and Tpk3p were compromised in the screen. Nonetheless, because of the extent to which

Hrr25^{Degron} depletion affects Nup53p phosphorylation, and the observed physical interactions between these proteins (Fig. 5-5 and 6), it is likely that Hrr25p makes a significant contribution to the observed phosphorylation of Nup53p.

Only two substrates of Hrr25p, Swi6p and Crz1p, are known (Ho et al., 1997; Kafadar et al.,. 2003; Ubersax et al., 2003). Both Swi6p and Crz1p are transcription factors that control the expression of genes transcribed in response to DNA damage or to elevated intracellular Ca^{2+} levels, respectively. The addition of Nup53p to this list suggests that Hrr25p is involved with regulating a number of processes. This idea is reflected in the diverse phenotypes of *HRR25* mutants, which have been shown to have defects in DNA repair, calcium regulated pathways, and the secretory pathway (Ho et al., 1997; Murakami et al., 1999; Kafadar et al., 2003). They also exhibit obvious defects in cell cycle progression, including aberrations in morphogenesis, nuclear division, cytokinesis and meiosis, suggesting that Hrr25p may function as a cell cycle regulatory kinase (Hoekstra et al., 1991).

Our analysis of the localization of Hrr25p supports the idea that Hrr25p plays an important role in controlling cell growth. Unlike previous studies, we investigated the localization of Hrr25p from a cell cycle perspective, monitoring a functional GFP-tagged version of the endogenous protein in synchronous live cell cultures. This approach revealed that Hrr25p is an extremely dynamic protein that localizes to regions of active cellular growth including the bud tip, bud cortex, and bud neck (Fig. 5-7). While these distributions are consistent with those reported by other groups (Vancura et al., 1994; Kafadar et al., 2003), we also uncovered a previously unidentified localization of Hrr25p at spindle pole bodies (SPBs). This distribution is particularly striking during mitosis and

is similar to the localization of a mammalian orthologue, α-CK1. α-CK1 is localized at centrosomes and during mitosis also distributes to the mitotic spindle, including those microtubules that attach to kinetochores (Brockman et al., 1992). The functional significance of this localization is still unknown, but it may be important for regulating progression into mitosis (Gross et al., 1997). We did not detect a similar kinetochore distribution for Hrr25p, however, our data cannot exclude this possibility as SPBs and kinetochores are difficult to resolve by light microscopy in yeast (Jin et al., 1998). Regardless, as mitosis draws to a close, Hrr25-GFP redistributes from SPBs to the bud neck in a pattern that resembles septin rings. This change in localization is similar to that observed for a number of kinases important for mitotic progression and mitotic exit, including Cdc5p, Cdc15p and Dbf2p, and provides further support for the idea that Hrr25p is an important mitotic regulator (Cid et al., 2002).

Our results establish that Nup53p is able to interact with Hrr25p both *in vitro* and *in vivo*, yet we did not observe Hrr25-GFP concentrating at the NPC at any point during the cell cycle. It remains a possibility, however, that the nuclear accumulation of Hrr25-GFP masked its association with the NPC. Interestingly, the loss of the nuclear accumulation of Hrr25-GFP occurs at a time point that correlates with Nup53p phosphorylation, and the attenuation of the physical interaction between Hrr25p and Nup53p (Fig. 5-6 and 5-7). These data are consistent with a model whereby Hrr25p phosphorylates Nup53p as it shuttles out of the nucleus (Fig. 5-9). Alternatively, it is also possible that the phosphorylation of Nup53p may play a direct role in the movement of Hrr25p out of the nucleus. A prediction of this model is that the phosphorylation of Nup53p would destabilize its interaction with Hrr25p. Our results support this idea, as

we observe that the interaction between Nup53p and Hrr25p is less stable when Nup53p is phosphorylated (Fig. 5-5). One could envision that the phosphorylation of Nup53p might serve to temporally regulate the nuclear distribution of Hrr25p. In this scenario, a pool of Hrr25p could be retained in the nucleus due, at least in part, to its interaction with Nup53p (Fig. 5-9). Once Nup53p is phosphorylated, Hrr25p would be free to shuttle out through the NPC. This potential builds on our previous proposal that the NPC can function to temporally control the localization of key cell cycle regulators. For example, we have proposed a similar function for the NPC in regulating the localization of the SAC protein Mad1p (Scott et al., 2005 and see section 6.7, also Fig. 5-9).

We detect the phosphorylation of Nup53p throughout mitosis, but it occurs most strikingly in cells arrested in metaphase (Fig. 5-1). Unlike in synchronous, unarrested cell cultures, the metaphase-specific phosphorylation of Nup53p appears to mirror the rise in levels of the mitotic cyclin, Clb2p. This suggests that there is a strong correlation between Cdk1p/Clb2p activity and Nup53p phosphorylation. Consistent with this idea, we also provide evidence that Cdk1p phosphorylates Nup53p (Fig. 5-2). While *in vitro* evidence suggests that Cdk1 phosphorylates certain vertebrate nups (Macaulay et al.,1995; Favreau et al., 1996), our data are the first *in vivo* evidence of such a role. It is likely that Cdk1p targets a number of nucleoporins in both yeast and vertebrates. The recent proteomic analysis that helped us identify Nup53p as a Cdk1p substrate also revealed that Nup60p and Nup1p, two nuclear basket nups, are also *in vitro* targets of this kinase (Ubersax et al., 2003). In addition, there is evidence in *N. crassa* that Cdk1 may play a role in the phosphorylation of nups, and that these events may be required for

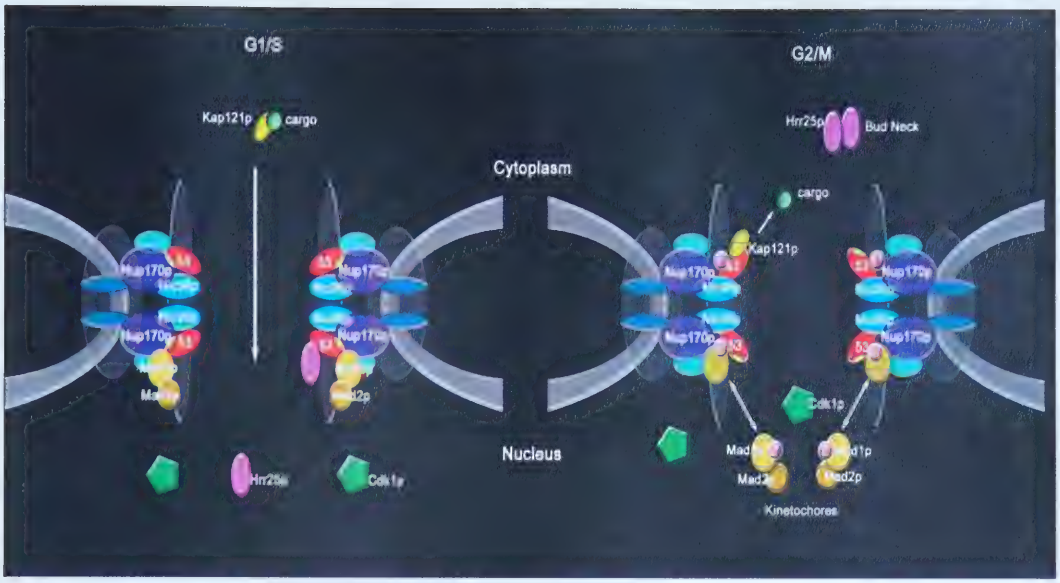

Figure 5-9. A speculative model for the potential functions of Nup53p phosphorylation.

During G1/S-phase, Nup53p interacts with Nup170p, hiding the Kap121p binding domain (yellow oval) and allowing the import of Kap121p (yellow) and its cargo (green sphere). We also detect an interaction between Nup53p and Hrr25p (purple) and we propose that this association may play a role in maintaining Hrr25p in the nucleus. Mad1p (yellow) and Mad2p are associated with the Nup53p subcomplex (Iouk et al., 2002), likely through a direct interaction between Mad1p and Nup53p (Scott et al., 2005). Upon spindle checkpoint activation, cells arrest in G2/M. Hrr25p and Cdk1p (green) both phosphorylate Nup53p (P). The phosphorylation of Nup53p may function in a number of capacities. It may drive the molecular rearrangments that expose the Kap121p binding domain which inhibits Kap121p-mediated import. Alternatively, it may contribute to the dissociation of Mad2p and a portion of Mad1p from the NPC where they are targeted to kinetochores, and thus function in the spindle assembly checkpoint. We also propose that the phosphorylation of Nup53p by Hrr25p allows Hrr25p to exit from the nucleus where it can be targeted to the bud neck.

progression from G2 to M-phase (Wu et al., 1998; De Souza et al., 2003; De Souza et al., 2004). Our data does not support a similar role for the phosphorylation of Nup53p at the G2/M transition, as mutation of the two Cdk1p consensus sites does not appear to affect the growth rate of these strains on plate media, at least within the context of a *nup53Δ* (Fig. 5-8B, C, *nup53Δ* panels). However, it remains a possibility that Hrr25p activity can functionally substitute for Cdk1p as it too has been suggested to act at the G2/M transition, perhaps upstream of Cdk1p (Hoekstra et al., 1991).

At present, we cannot distinguish between the relative contributions of Hrr25p and Cdk1p to the electrophoretic mobility shift of Nup53p, as both the inhibition of cdk1-as1 and the depletion of Hrr25p^{Degron} have similar effects on the visible phosphorylation of Nup53p (Fig. 5-2A and 5-4B). One could envision that these kinases act simultaneously to phosphorylate Nup53p, or that they act sequentially. Consistent with the latter idea, casein kinases phosphorylate amino acid residues C-terminal to acidic amino acid residues or previously phosphorylated sites (Flotow et al., 1990; Flotow and Roach, 1991). However, of the 10 putative casein kinase sites in Nup53p, none appear to be proximal to a Cdk1p site. Therefore, to fully differentiate the relative contributions of these two kinases will likely require the identification of the specific amino acid residues that are modified by each kinase. The identification of these kinase targets will also be critical to understanding the function of Nup53p phosphorylation.

In Chapter IV we showed that during mitosis Nup53p's interactions at the NPC are altered such that a cryptic binding site for the karyopherin Kap121p (the KBD) is revealed, inhibiting nuclear import of this transport pathway. We hypothesized that it was the phosphorylation of Nup53p that would bring about these rearrangements and the

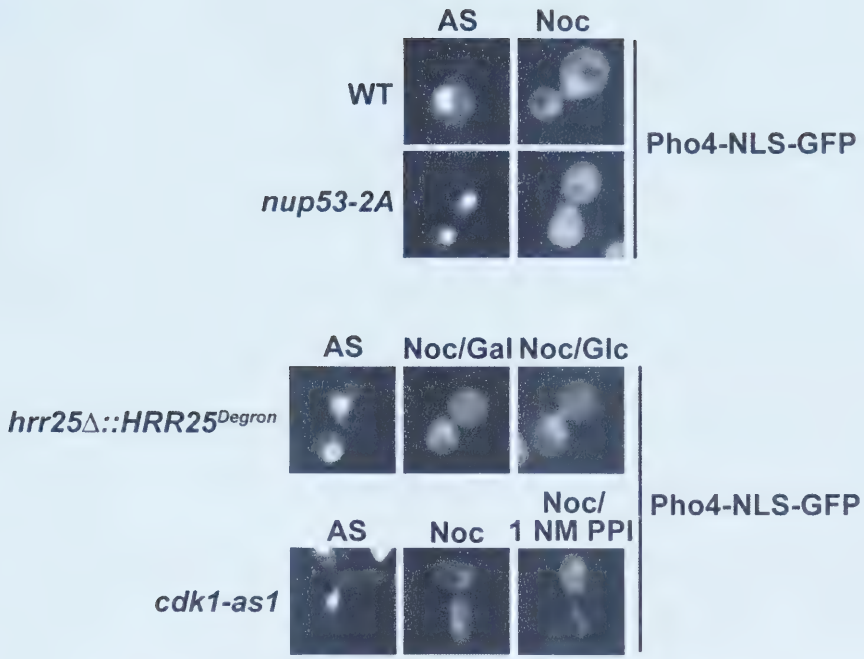

Figure 5-10. Kap121p import remains inhibited in kinase mutants.

The distribution of Pho4-NLS-GFP was monitored in asynchronous (AS) or nocodazole arrested cultures (Noc) in the following strains by fluorescence microscopy: BY4741 (WT), CPL66 (*nup53-2A*), KKY387 (*HRR25*^{DEGRON}) and DOMOO39 (*cdk1-as1*). To deplete Hrr25p^{Degron} cells were transferred from galactose (gal) to glucose containing media (Glc). To inhibit cdk1-as1, 10 μg/ml 1 NM PPI was added to nocodazole arrested cells for 10 minutes before image acquisition.

resulting import inhibition. However, depleting Hrr25p^{Degron} or inhibiting cdk1-as1 did not reverse this inhibitory pathway, suggesting that either kinase may be sufficient to induce key NPC rearrangments, or that they may be triggered by other modifications of Nup53p (Fig. 5-10 and see section 6.4).

Alternatively, the phosphorylation of Nup53p could function as a part of the spindle assembly checkpoint (SAC) response. The SAC ensures the proper timing and fidelity of chromatid separation at the metaphase-anaphase transition (Lew and Burke, 2003) and Nup53p physically interacts with a central player in this pathway, Mad1p. Mad1p is localized predominantly at NPCs, and this distribution is dependent on a number of nups, including Nup53p (Iouk et al., 2002; Scott et al., 2005). In addition, Mad1p is not phosphorylated upon SAC activation in the absence of Nup53p (Iouk et al., 2002). This suggests that Nup53p may function during the SAC, and consistent with this idea, we visualize the most striking forms of Nup53p phosphorylation in cells arrested in metaphase (Fig. 5-1 and 5-2). Furthermore, recent data suggest that although Nup53p may not be required for SAC activation, it may be involved with modulating the fidelity of the checkpoint response (Scott et al., 2005). This phenotype is thought to be related to the relative resistance that *NUP53* mutants display for growth on media containing the microtubule destabilizing drug benomyl (Iouk et al., 2002; Scott et al., 2005). A similar benomyl resistance is displayed by mutants in *NUP170*, which could suggest that Nup53p and Nup170p have interdependent functions with respect to SAC function. Consistent with this idea, the combination of null strains of both *NUP53* and *NUP170* results in synthetic lethality (Marelli et al., 1998). Our results indicate that the Cdk1p phosphorylation mutant, *nup53-2A*, does not retain the functionality of Nup53p in the

absence of Nup170p. This is particularly true in the presence of benomyl where *nup53-2Δnup170Δ* strains exhibit a marked sensitivity to benomyl, similar to that observed for strains with lesions in the SAC (Lew and Burke, 2002; Iouk et al., 2002; Scott et al., 2005). These data are consistent with a model in which Nup53p phosphorylation is required, in the absence of Nup170p, to maintain a SAC arrest upon microtubule destabilization. We propose that the phosphorylation of Nup53p may play a role in regulating the association of Mad1p and Mad2p with kinetochores, although clearly further experiments are required to test this hypothesis (Fig. 5-9). This putative Nup53p function, however, may be relevant to a conserved functional role for Nup53p as, unlike the KBD which appears to be absent, Nup53 maintains its interaction with Mad1 in human cell lines (Marelli et al., 1998; Hawryluk-Gara et al., 2005).

Chapter VI: *Perspectives*

6.1 Synopsis

Nup53p was initially identified in complex with a number of nucleoporins including Nup170p and Nup59p, in addition to the kap Kap121p (Marelli et al., 1998). This work establishes further functional links between these proteins including a role for kaps in assembling the NPC, and the existence of a mechanism to inhibit Kap121p transport during mitosis. We also establish new insights into the proteins regulating the phosphorylation of Nup53p. In this chapter, this work will be discussed within a broader and speculative context, and provide alternative explanations for some of the results. In so doing, I hope to leave the reader with a sense of where I feel the future of Nup53p, and NPC biology as a whole, are evolving.

6.2 Kaps as chaperones

To efficiently assemble a NPC requires both a spatial and temporal regulation. As a part of this regulation, there is accumulated evidence that supports the idea that nups are sequentially recruited to a growing NPC in a defined order (Bodoor et al., 1999; section 1.8). The mechanisms that control this order are in large part unknown, but there is a growing perception based on our work and others' that kaps may function in this capacity. Consistent with this idea, kaps regulate NPC assembly in *Xenopus* extracts at numerous steps that have been linked with their cargo carrying capacity, their interactions with FG and non-FG nups and Ran-GTP (Harel et al., 2003; Walther et al., 2003; Zhang et al., 2002; Hachet et al., 2004). For example, kaps have been proposed to prevent the assembly of nups until they encounter Ran-GTP, which is thought to release this inhibition and allow nups to form sequential and higher order interactions (Walther et al.,

2003). This kap function is analogous to that of chaperones which help build complexes by preventing the premature aggregation of proteins, a process which is generally regulated by ATP-hydrolysis (Agashe and Hartl, 2000; Jakel et al., 2002). We proposed the idea that Kap121p may act as a chaperone to bring Nup53p into the context of the NPC and maintain it in a conformation competent to bind Nup170p (see section 3.6). Others have expanded on this notion. Jakel et al. (2002) provide evidence that kaps act as chaperones by protecting basic patches on cytoplasmic proteins from aggregating. A similar function may also be important for nuclear transport, as shielding basic regions from the hydrophobic central channel may be critical for efficient translocation (Ribbeck and Gorlich, 2002).

If kaps have the ability to regulate nup-nup interactions during assembly, it logically follows that kaps may alter nup-nup interactions within a fully formed NPC. This hypothesis is featured in a number of transport models. For example, to solvate into the "selective phase" requires the local disruption of FG-FG interactions by the kap-cargo complex (section 1.5). Another model predicts that kaps act as catalysts to break open a "metastable network" of nup-nup interactions which form the diffusional barrier imposed by the NPC (Kustanovich and Rabin, 2004). Such a mechanism may also be relevant for transport models that consider the central channel to be gated (Shulga and Goldfarb, 2003), whereby kaps could gain access by breaking nup interactions to open the channel. Kap121p may perform a similar function for Nup53p, that is, the binding of Kap121p to Nup53p could alter its interactions with nups. This is clearly the case *in vitro*, as Kap121p can disrupt interactions of Nup53p with Nup170p (Fig. 3-8). These results

might provide an explanation for how Nup53p is able to interact with so many proteins in a regulated fashion (see below).

6.3 Nup53p organization in the NPC

Based on our interaction data, Nup53p is able to interact directly with a number of proteins including Nup170p, Nup59p, Nic96p, Kap121p, Hrr25p, Mad1p and potentially Ndc1p and Nup192p (Marelli et al., 1998; Uetz et al., 2000; Scott et al., 2005; see Appendix, Fig. 8-1). Nup53p is also able to associate with the inner nuclear membrane (Marelli et al., 2001). It isn't probable that Nup53p could simultaneously interact with all of these proteins, even within the context of two separate complexes. I suggest that Nup53p is organized in one of two ways. Either small pools of Nup53p stably interact with multiple partners, perhaps in multiple subcomplexes, or, Nup53p temporally interacts with specific proteins in response to environmental cues or cell cycle progression (Chapter IV). Our data would support the latter idea, since during mitosis we are unable to detect any Nup53p in association with Nup170p, or during S-phase with Kap121p (Fig. 4-4 and Fig. 3-8). This suggests that there is an "all or nothing" interaction between at least a pool of Nup53p, Nup170p and Kap121p. As I alluded to above, the interaction with Kap121p could function alongside other modifications such as phosphorylation to modulate the interactions between Nup53p and different nups. This hypothesis might predict that the pool of Nup53p that interacts with Kap121p may be transiently mobile within the context of the NPC. If such a pool of Nup53p exists, then a prediction of this model is that mutations in Kap121p would alter the distribution of Nup53p (Fig. 3-4). We have interpreted the mislocalization of Nup53p in *kap121*

mutants as a defect in the assembly of Nup53p into the NPC (Chapter III). Our data, however, equally supports the idea that Kap121p may function to tether a fraction of Nup53p at the NPC. Similar interpretations have been made regarding the role of Kap121p and Kap95p in the NPC localization of Ulp1p, in addition to the role that Kap95p plays in the localization of Mad1p (Panse et al., 2003; Scott et al., 1005). In further support of this model, evidence for a kap-tethering mechanism has been predicted from *in vitro* data, whereby an interaction between Nup100p and Nup2p can be bridged by the Kap95p/Kap60p heterodimer (Allen et al., 2002).

6.4 The inhibitory signal

Whether Kap121p modulates these interactions or another mechanism is involved, our data strongly support that there are changes in the interactions between Nup53p and its binding partners during mitosis (Chapter IV). We hypothesized (section 4.7) that the phosphorylation of Nup53p was the driving force behind these rearrangements, but we were not able to confirm this idea since we could not reverse the import inhibition in our kinase mutants (Fig. 5-9). By overlay assay, we were also unsuccessful in detecting any differences in the interaction between Nup170-pA, Kap121-pA and recombinant Nup53p phosphorylated by *in vitro* kinase reactions (Appendix, Fig. 8-2). These data cannot completely rule out the possibility that phosphorylation plays a role in modulating these interactions, but they clearly do raise the question of whether there are other mechanisms at work. One other posible regulator of Nup53p interactions is sumoylation. In the last few years there have been numerous functional links established between the sumoylation machinery and the NPC (reviewed in Melchior et al., 2003). A case in

point, Nup358 has been recently shown to play a direct role in the conjugation of SUMO on proteins (Pichler et al., 2002). In addition, a recent proteomics screen for sumoylated proteins identified a number of nups that were sumoylated including Nup98 and Nup96 (Li et al., 2004). Nup53 was not identified in this screen but we have identified two consensus sumoylation sites in Nup53p that can be sumoylated *in vitro* (J.D. Aitchison, personal communication). These sites are located at amino acid residues 415 and 423, conspicuously in the middle of the KBD.

6.5 Nuances in nuclear transport

Whether it is sumoylation, phosphorylation or another mechanism, our data support a model whereby molecular rearrangements within Nup53p's interactions occur to inhibit Kap121p-mediated import during mitosis. Our mechanism is based on the revealing of a high affinity kap binding site within the central channel that inhibits Kap121p translocation and displaces cargo (Fig. 4-7). On the surface, this model is compatible with most current transport hypotheses, which require low affinity interactions – at least on the cytoplasmic side of the NPC - to facilitate transport (section 1.5). The one possible exception is the Virtual Gate (1.5.2) which proposes that the greatest barrier to macromolecular transport is an entropic barrier imposed by the size of the transport channel (Rout et al., 2000; Rout et al., 2003). Our model predicts that cargo would be released within the central channel, and should have therefore overcome this barrier. It is extremely difficult, however, to suggest that this result might be inconsistent with this transport model. If nothing else, it serves to reveal the complexity of nuclear transport and how current models must incorporate mechanisms that allow for the

regulation of individual transport pathways. Furthermore, it may serve to emphasize that to fully understand transport may require the imaging of individual cargos and kaps in transit through the NPC (Yang et al., 2004; Babcock et al., 2004; Kubitscheck et al., 2005). Future transport models will likely rely heavily on data from these types of experiments in addition to the reliance on more computational and mathematical modeling to derive insight into this process (Becskei and Mattaj, 2004).

6.6 Towards atomic resolution

While this kind of analysis will be helpful, to understand nuclear transport will require more details regarding the nature of the biochemical interactions between individual nups and how they form an active transport channel. Ultimately this will require an atomic resolution model of the NPC. As it stands, we have likely imaged the NPC at the limits of EM-resolution with the recent work of both Stoffler et al. (2003) and Beck et al. (2004). Visualizing the organization of individual subcomplexes has met with some success; but are still limited by EM-resolution (Siniossoglou et al., 2000; Lutzmann et al., 2002). Atomic structures of nups have proven elusive, likely because most are highly insoluble and have unfolded domains which resist crystallization (Denning et al., 2003; Devos et al., 2004). Nonetheless, three groups have persevered in crystallizing domains of Nup98, Nup133 and Nup159p (Hodel et al., 2002; Berke et al., 2004; Weirich et al., 2004). The structures of both Nup133 and Nup159p reveal a common structural fold consisting of a series of β-sheets that form into a multi-bladed propeller. This fold is predicted to exist in about a third of all nups (Berke et al., 2003) and underscores the notion that the NPC is constructed by a few repetitive building blocks. Consistent with

this idea, β-propellers have numerous surfaces that can mediate protein-protein interactions. Intriguingly, they are also remarkably similar to domains found in clathrin and vesicular coat proteins. It has been hypothesized that within the context of nup structure, these domains may function to bend the nuclear envelope and form the pore membrane, using mechanisms analogous to those that form coated vesicles (Devos et al., 2004). This hypothesis has yet to be directly tested.

6.7 The NPC hub

The β-propeller domain in Nup159p has also been implicated in mediating an interaction with an mRNA helicase, Dbp5p, that functions during mRNA export (Weirich et al., 2004). The interaction of an mRNA helicase with the NPC illustrates what is becoming a common theme in NPC biology. The NPC appears to function as a host for proteins that are not bona fide nups, but nonetheless distribute predominantly to the NPC. We have proposed that the NPC can temporally sequester these proteins until they are needed at other cellular locales (Scott et al., 2005). In this fashion, the NPC could potentially regulate a number of diverse cellular processes outside of nuclear transport. This hypothesis is founded on the dynamic localization of Mad1p and Mad2p, two SAC proteins with the NPC. Upon SAC activation pools of these proteins are released from the NPC and localize at kinetochores (Campbell et al., 2001; Iouk et al., 2002; Gillett et al., 2004; Scott et al., 2005). How the NPC regulates the release of these proteins is not known, although the interaction with Mad1p and the NPC is determined by a number of proteins including Nup53p, Nup60p and Kap95p (Scott et al., 2005). Mutations in these components affect the fidelity of the spindle checkpoint response.

Other examples include members of the sumoylation machinery. The distribution of Ulp1p/SENP2, a protease that processes SUMO and desumoylates proteins, must be tightly regulated (Panse et al., 2003; Zhang et al., 2002; Li and Hochstrasser, 2003). Delocalizing Ulp1p from the NPC can have toxic effects on cells including defects in DNA repair (Zhao et al., 2004) and in some cases, cell death (Panse et al., 2003). Moreover, data from our lab suggest that Ulp1p may regulate the desumoylation of septins, which would require a regulated release of Ulp1p into the cytoplasm at the end of mitosis. Consistent with this idea, mutations in Kap121p that partially mislocalize Ulp1p result in the premature desumoylation of septins (T. Makhnevych and R.W. Wozniak unpublished data). Other members of the sumoylation machinery also reside at the NPC, including the sumoylating enzyme Ubc9p (Zhang et al., 2002; Lee and Melchior 1998; Panse et al., 2003) which functions in conjunction with Nup358/RanBP2 (Pichler et al., 2002). These proteins may have targets outside of the NPC, but they are also required for the nuclear import of some proteins (Stade et al., 2002: Pichler and Melchior, 2002). This function might serve to highlight that the NPC distribution of these proteins may also contribute to nuclear transport. Mad1p appears to function in a similar capacity as *mad1Δ* strains have defects in the nuclear import of an NLS reporter (Iouk et al., 2002).

6.8 Nup53p and the SAC

Mad1p directly interacts with Nup53p and this interaction has formed the basis of two studies investigating functional links between Nup53p and the SAC (Iouk et al., 2002; Scott et al., 2005). In terms of this thesis, the NPCs putative role in the SAC may

be particularly relevant to the delay in the metaphase-anaphase transition we observe upon overexpression of *NUP53* (Fig. 4-6). In Chapter IV, we proposed that the overexpression of *NUP53* inhibits Kap121p mediated import which results in a phenotype similar to that which is observed in strains harboring the *kap121-34* allele (Fig. 4-1). It is equally likely, however, that the delays in mitosis caused by *NUP53* overexpression are functionally linked to the spindle assembly checkpoint. Indeed, recent data suggest that the delays in Clb2p degradation that we observed upon *NUP53* overexpression are dependent on Mad1p and therefore the SAC machinery (T. Makhnevych and R.W. Wozniak, unpublished data). Even this idea, however, is not entirely supported by the data since the degradation of the anaphase inhibitor, Pds1p, occurs normally (Fig. 4-6). This is unexpected, as the activation of the SAC results in a stabilization of Pds1p which inhibits chromatid separation (Lew and Burke, 2002). Our data are therefore more consistent with a delay that occurs after chromatid separation, and might be similar to the phenotypes observed in cells with defects in proteins required for spindle elongation (Pellman et al., 1995; Saunders et al., 1995). While this is clearly speculation, functional links are growing between the spindle and the nuclear transport machinery (see Dasso, 2002), including the Nup53p complex. This includes the observed growth resistance to microtubule destabilizing drugs observed in nup knockout strains, particularly those of the Nup53p subcomplex, and an observed functional interaction between Nup53p and Bim1p (Iouk et al., 2002; Scott et al., 2005; Quimby et al., 2005; and Appendix, Fig. 8-3). Bim1p is a microtubule binding protein involved with nuclear migration during mitosis (Miller et al., 2000).

6.9 The NPC and the cell cycle

There are numerous ways in which the NPC could influence spindle dynamics. In organisms with a closed mitosis, this could include the regulation of the nuclear import of key regulators of this process including tubulin and Cdk1p (Ovechkina et al., 2003; Wu et al., 1998). Alternatively, components of the NPC could directly interact with the spindle. Nup358/RanBP2 distributes to both the spindle and kinetochores in vertebrates (Joseph et al., 2002; Joseph et al., 2004) and the knockdown of Nup358 by RNAi causes dramatic defects in kinetochore structure and the mislocalization of kinetochore and spindle checkpoint proteins (Salina et al., 2003; Joseph et al., 2004). Furthermore, a pool of the Nup107-160 complex distributes to kinetochores during mitosis, although the functional significance of this event remains entirely unknown (Belgareh et al., 2001; Loiodice et al., 2004). Addressing this putative function might prove easier in yeast, but these nups have not yet been observed in association with yeast kinetochores. Nonetheless, there are overlaps between the NPC and the cell cycle machinery in yeast as well.

The distribution of Mad1p and Mad2p with the NPC is one example of the overlap between the cell cycle machinery and the NPC in yeast (see above), but another is Ndc1p. Ndc1p is a shared component of NPCs and spindle pole bodies (SPBs) where it plays a role in SPB insertion into the NE (Chial et al., 2000). Interestingly, Ndc1p interacts with Nup53p by two-hybrid analysis which could suggest that a fraction of Nup53p may also localize to SPBs (Uetz et al., 2000). Consistent with this idea, Nup53p was identified in complex with the SPB protein Tem1p in a large scale proteomics analysis of protein complexes (Ho et al., 2002). This potential localization of Nup53p

may also be reflected in the SPB localization of Hrr25p which occurs when Nup53p becomes phosphorylated (Fig. 5-7).

The overlap of nups with the cell cycle machinery, particularly in vertebrates, intimately links the mechanisms governing NE breakdown (NEBD) and reassembly with the proper segregation of chromosomes. The timing of these events must be tightly regulated and sharing components may provide an efficient way to maintain this regulation. This hypothesis has yet to be directly tested, but functional links between the spindle and NEBD have been described. NEBD is facilitated by the early precursor of the mitotic spindle which physically tears the NE apart (Beaudouin et al., 2002; Salina et al., 2002). It has been hypothesized that NPCs may provide the anchor points for microtubule motors to facilitate this process (Salina et al., 2002; Beaudouin et al., 2002), again illustrating the growing functional diversity of the nuclear pore complex.

Chapter VII: *References*

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Chapter VIII: *Appendix*

The Appendix contains the results of a number of experiments that are unpublished, and are not a part of the results sections in the previous chapters. They consist of a series of *in vitro* binding experiments where Nup53p is shown to directly bind to GST-fusions of Nup170p. Nic96p and Nup59p (Fig. 8-1). Fig. 8-2 displays results acquired from overlay experiments where whole cell extracts prepared from yeast strains producing Kap121-pA and Nup170-pA were used to probe a nitrocellose membrane containing recombinant purified Nup53p. An adjacent lane contains Nup53p that has been phosphorylated by an *in vitro* incubation with a mitotic *Xenopus* extract. Lastly, Fig. 8-3 shows the growth characteristics of yeast strains containing deletions of *NUP53* and *BIM1* grown on benomyl containing plates.

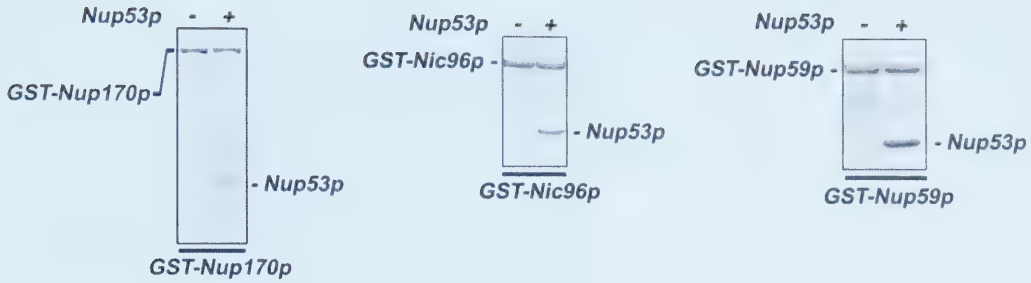

Figure 8-1. Nup53p interacts directly with Nup170p, Nup59p and Nic96p.

Recombinant GST-fusions of the indicated nups were immobilized on GT-Sepharose and incubated (+) or mock incubated (-) with recombinant, purified Nup53p. After washing, bound fractions were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and visualized using Coomassie staining.

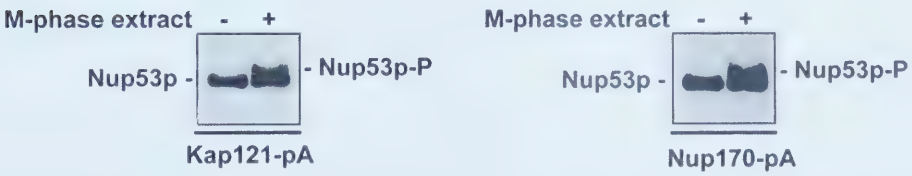

Figure 8-2. Nup170-pA and Kap121-pA interact with phosphorylated Nup53p.

Purified, recombinant Nup53p was mock incubated (-) or incubated (+) with M-phase *Xenopus* extracts to drive the phosphorylation of Nup53p. Reaction products were separated by SDS-PAGE and immobilized on nitrocellulose. Extracts from yeast strains producing protein A fusions of Nup170p (NP170pA) or Kap121p (KP121pA) were used to probe the membranes. Protein A fusions were detected using HRP-conjugated IgG and ECL.

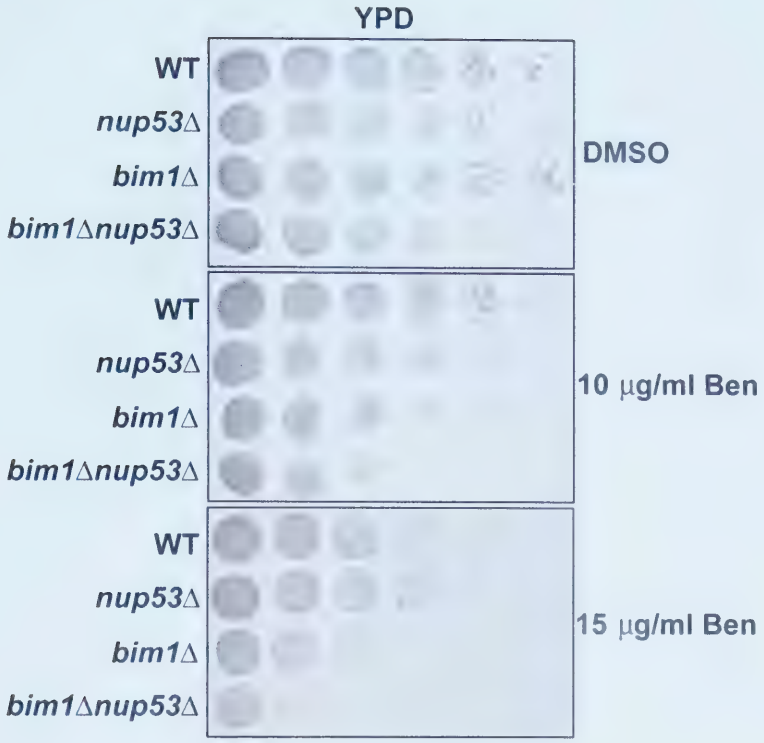

Figure 8-3. *bim1Δnup53Δ* strains exhibit heightened sensitivity to benomyl

A wild-type (WT) yeast strain (BY4741) and the indicated null strains (nup53Δ, bim1Δ, and CPL75) were grown to mid-log phase and then spotted in 10-fold serial dilutions on enriched plated media (YPD) containing carrier (DMSO) or benomyl (Ben) at the indicated concentrations. Plates were grown from 3-5 days at 25°C.

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